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THE RELATION OF BORON TO THE ROOT-NODULES OF VICIA FABA

By

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(*Received for publication October 14, 1940*)

It has been known for some years that boron is essential for the growth of certain leguminous plants, but its relation to the cytology of the nodule has awaited investigation.

Miss Warington (1923) described the effects of boron deficiency on *Vicia Faba* grown in solution culture, and McHargue (1940) commented on the high content (30 p. p. m.) of boron in leguminous hay in contrast with a smaller amount (5 to 10 p. p. m.) in grass hay. Dufrenoy (1937) traced a disease of *Vicia Faba* in southwestern France to boron deficiency and Bruneteau (1939) demonstrated that applications of boron to the soil were beneficial in such cases. Brenchley and Thornton (1925) showed that the growth of nodule bacteria in pure cultures was independent of the presence of boron, likewise the early development of nodules on the roots. The formation of a vascular system, connecting the meristematic cells in the nodules with the vascular system of the mother root, depends, however, upon the presence of boron. Dufrenoy (1938) found that, as a result of boron deficiency, nuclear divisions may lag behind cell divisions. Chen and Thornton (1940) correlated the nitrogen-fixing efficiency with the volume of cells which became infected, and with the time that elapses before these cells collapse and disintegrate.

The following studies were made on *Vicia Faba* plants collected in February from a number of fields in southwestern France on a silt soil having a low base-exchange capacity. The local designation of the soils is "Boulbenes". The plants taken from these fields had made poor growth, their internodes were short, they formed a small amount of seed, and their terminal buds became necrotic.

The collected plants were carefully transplanted to the laboratory, with balls of soil around their roots, and put into 8-inch paraffined earthen pots. Two plants from each field were thus installed. One plant of each pair received 1 gram of boric acid, the other none. Two plants from each of the same fields were carefully removed from the balls of soil and were transferred to Pfeffer's solution in paraffined glass jars. One plant of each pair in these solutions received 1 part per 1000 of boric acid, the other none. The amounts of boric acid added may seem large in comparison with that employed by other workers, but adult plants, due to their greater carbohydrate supply, are less susceptible to boron than young plants.

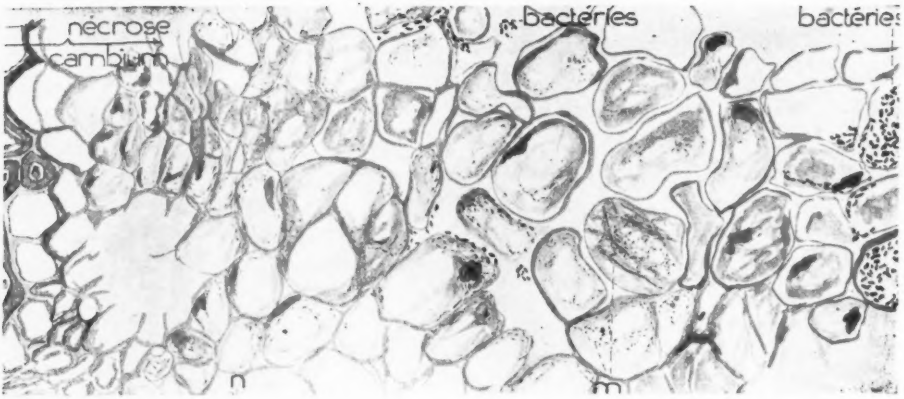

Fig. 1. Section of nodule at its insertion on root of *Vicia Faba* grown in Pfeffer's solution without boron; killed in Helly fluid, stained with acid fuchsin and methyl green. Extensive necrosis is evident at the left part of the figure in the immediate vicinity of the wood vessels. Infected cells of the nodule tissue are seen at the right of the figure. Bacteria from the infected cells have invaded a number of cells towards the left; most of the infected cells have already collapsed, many show various stages of proteolysis; a few remain turgescent and show mitochondria.

In the presence of boron, in soil and solution cultures alike, the plants started a vigorous vegetative growth, developed axillary buds, blossomed and produced pods. In the absence of boron the plants died back within three or four weeks. The symptoms of boron deficiency developed more rapidly when plants received additional illumination at night during the short days of February and March, as suggested by Warrington (1933).

Fig. 2. A turgescent cell containing long mitochondria from nodule of *Vicia Faba* grown without boron, examined vitally in Ringer solution.

Fig. 3. An uninfected cell, showing mitochondria, between two infected cells, full of bacteria, from a nodule of *Vicia Faba* grown in Pfeffer's solution plus 1 part per thousand boric acid; killed in Helly fluid, stained with acid fuchsin and methyl green.

Root-nodules were collected and examined at intervals after the plants were installed in the experiment. The sections of nodules showed that bacteria were released from the infection threads into the cytoplasm of the host cells with less tendency to swell in the absence of boron, than bacteria in the corresponding tissues of nodules of plants which received boron.

In the nodules of boron-deficient plants, the bacteria spread towards the vascular tissue of the parent root, and eventually the endodermis, pericycle, and phloem tissues exhibited necrosis (Fig. 1). Nodule cells infected by the bacteria collapsed and disintegrated after a time whether the plants received boron or not. In plants receiving boron, such cells collapsed after having functioned efficiently in nitrogen fixation; while without it, they collapsed prematurely. Uninfected cells remained turgid, and their pressure on the hypoplastic infected cells caused them to collapse. When this process had advanced, the appearance of a section suggested intercellular infection (cf. Thornton, 1940). This was especially true for nodules of plants grown without boron, where infected cells soon disintegrated, leaving pockets crowded with bacteria. A few cells, apparently uninfected, survived, in which there was an impressive array of mitochondria many of which were short rods and others were long flexous threads (Figs. 1 and 2).

Symptoms of recovery in the nodule tissues could be observed before there was any amelioration of the aerial parts, after the addition of 1 part per 1000 boric acid to the Pfeffer solution in which plants which had been formerly starved for boron had been placed. In those cases disintegration was checked and healthy growth of the cells was resumed.

Infected cells of roots which received boron disintegrated only after a period of activity, while the neighboring uninfected cells retained a high rate of metabolic activity as illustrated by Fig. 3. The upper part of the drawing shows rows of rod-shaped mitochondria in strands of cytoplasm outlining a number of small vacuoles. The lower part of the drawing shows very short mitochondria, chiefly in the region of the bacteria. Numerous examples of this phenomenon are known, and it seems to be a typical response of the cell to stimuli arising from bacteria, fungi, viruses, or nutritional disturbance. (Reed and Dufrenoy, 1935). In other nodules, mitochondria differentiated into amyloplasts and accumulated starch. Reed and Dufrenoy described similar cases and showed that they were correlated with an inhibition of cell division in cells which would otherwise have remained meristematic. Boron deficiency according to Brenchley and Thornton (1925) may also deprive the nodule bacteria of an adequate supply of carbohydrate, thereby unbalancing their nutritive relations and causing them to become parasitic on the cytoplasm of the host cells.

SUMMARY

The symbiotic relations between the nodule bacteria and their host cells are affected by the supply of boron. The behavior of the mitochondria and the effects of the bacteria on the continuance of the host cells are evidence of the importance of boron for the balance between the partners in symbiosis. A deficiency in a mineral nutrient like boron may cause the invading organisms to live as parasites.

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THE INFLUENCE OF l-PROLINE ON STRUCTURAL DIFFERENTIATION IN THE REGENERATING RIGHT CHELA OF THE HERMIT CRAB (PAGURUS LONGICARPUS)

By

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When it was found that l-proline provides a chemical grouping—presumably pyrrolidone—which acts to forward differentiation expression in Obelia (Hammett and Collings, 1937, and Hammett, 1938) further exploration was indicated. As part of the general plan experiments were run with the regenerating right chela of Pagurus. These are the subject of this report. The hermit crab claw was chosen because the regenerating member affords distinctive indices of structural differentiation and because it was desired to include the differentiation of regeneration in the final picture.

Five series of experiments were run. Each series started out with ten control animals, ten exposed to M/50,000 l-proline, and ten exposed to M/100,000. The three sets of each series were run simultaneously under like conditions of temperature, illumination, pH, and sea-water salinity. The right chela was removed by forceps as described by Hammett and Smith (1931). Procedures were in general the same as those described in that report. Quite small animals were used in order to foster quick regeneration of the new member. All were kept in fresh sea-water in fingerbowls, ten to a bowl The water was changed at least twice every 24 hours. Food was supplied from dark to dawn by live sand, small stones with barnacles, and sea lettuce. The l-proline was a Hoffman LaRoche product identified and recrystallized by Thomas Callan of The Institute Staff. The test crabs were exposed to the amino acid continuously from the beginning to the end of experiment. At the end of experiment the regenerated chelae were removed intact as described in the report of 1931. Four sets were fixed in Susa, cleared, stained with borax-carmine, and preserved in whole mounts in Canada balsam. The fate of these 120 regenerates is given in Table I. Measurements were made with a 10 mm. ocular micrometer divided into hundredths. The values given in the Tables are direct readings. Losses and mutilations were due to technical inexperience.

Comparison of the developmental expression of test and control claws can best be made from formed chelae in which the three segments used in this study can be clearly distinguished as shown by Plate 1. The three distal segments used here are designated as follows: the terminal segment, which carries the pincer, is called the Propus: the middle segment is named Dactylus: and the proximal segment, or the one nearest the body, is called the Merus. These segments are separated by chitinous septa from which the joints develop.

TABLE I

THE GROSS DEVELOPMENTAL STATUS OF THE REGENERATED CHELAE

	Controls		M/50,000		M/100,000	
Lost	1		8		1	
Mutilated	2		0		2	
		%		%		%
No Regeneration	1	2.7	1	3.1	0	0
Small Bud	1	2.7	1	3.1	0	0
Straight	4	10.8	2	6.2	1	2.7
One Bend	2	5.4	3	9.4	2	5.4
Propus:						
2.0 mm. or less	15	40.5	14	43.8	22	59.5
2.1 mm. or more	13	35.1	10	31.3	12	32.4
Heteromorphosis	1	2.7	1	3.1	0	0
Totals	40		40		40	

In any set of regenerating chelae there are some which do not regenerate during the time of observation. These are recorded in Table I as: No Regeneration. The chelae, like many other growths, start as small buds. Some of these also occur in most every set and are so recorded here. Then, some, for an unknown reason, fail to develop in the normal shape. They grow out straight instead of curving as shown on the Plate. These are designated as 'Straight'. A few grow straight but have the Propus bending but that is all. That is to say they present but one bend instead of three. They are so designated in the table. These then, are examples of unformed chelae in which structurel differentiation is either tardy or abnormal.

The formed chelae of these experiments were separable into two groups on the basis of average Propal length. One group was composed of those claws in which the Propus was 2.0 mm. or less in length: the other comprised the claws having a Propus which was 2.1 mm. or longer.

Fig. 1 Fig. 2 Fig. 3

Fig.4

Fig. 5

Fig. 1. l-Proline. 20 mm. set. Compare with Fig. 2 for Chitin and Pincer articulation. × 66.

Fig. 2. Control. 20 mm. set. × 66.

Fig. 3. l-Proline. 20 mm. set. Compare with Fig. 2 for Chitin, Septa, and beginning Pincer. × 66.

Fig. 4. Control. 21 mm. set. × 66.

Fig. 5. l-Proline. 21 mm. set. Compare with Fig. 4 for Chitin, Septa, and Pincer Development. × 66.

The table is not uninformative. The percentage of marked low development (first three classes) was greater in the controls. That is to say a higher percentage of claws in the l-proline series went on to good development. Moreover chitin thickness and septal growth were greater in the l-proline series despite the fact the regenerates were no larger. Turning now to the more natural and higher developed chelae it is seen that the l-proline series had a higher percentage of smaller (2.0 mm. and less) claws and tended to have a lower percentage of larger ones (2.1 mm. and over). These several differences suggest, but do not prove that progress of development was improved by l-proline.

Now this study is concerned, not with size differences, since these are largely conditioned by cell increase in number (Hammett and Hammett, 1932), but on the relative development of the l-proline and control chelae as expressed by their structural differentiation. It is obvious, other things being equal, that the larger the claw the more it is developed. This is visualized in the pictures of Plate 1, where Figures 4 and 5 are of larger chelae than those of Figures 1, 2, and 3, and where it is seen that the larger claws are much more differentiated than the smaller. *The comparison then is between control chelae of a given size, and those from the l-proline series of approximately equal or lesser average size.*

Table II gives the data from which estimate can be made. It omits all unformed chelae. Examples of formed chelae are given in the photographs. It records the dorsal length of the Propus, Dactylus, and Merus separately: the relative thickness of the enveloping chitin: the relative length and development of the septa which separate the segments: and the relative development of the pincer. Values are given for the four experiments separately and combined. All chelae in which the Propus was 2.0 mm. or less in length are in group 1: those in which the segment was 2.1 mm. or over are in group 2.

The recorded Propus length is the straight line distance from the tip to the dorsal Propus-Dactylus junction: the recorded Dactylus length is the straight line distance from the Propus-Dactylus junction to the Dactylus-Merus junction along the dorsum of the chela: and the recorded Merus length is the straight line distance from the Dactylus-Merus junction to the dorsal point of emergence of the Merus.

Since it was not feasible to make trial sections for planimeter determination of absolute values, the data for thickness of chitin; length and degree of development of intersegmental septa; and length and degree of pincer development are given as relative values only.

The regenerating chela is enveloped by chitin from the beginning. The thickness may vary from part to part, but is usually fairly uniform along the dorsum. It was here then that first estimate was made by ocular micrometer. Any distinctive accumulation or lack in other parts was used as adjunct to decision. The following classes of chitin thickness were recognizable. The class number is the value given to each. 1. Very Thin: 2. Thin: 3. Medium: 4. Thick: 5. Very Thick. The very thin was just a faint line of highly refractive material; the thin was approximately twice this and about 0.02 mm.; the medium was a well-formed layer close to 0.04 mm. thick; the thick was well over 0.05 mm. and the very thick close to and sometimes over 0.1 mm. Exact measurements were impossible.

TABLE II

BREAKDOWN ANALYSIS OF REGENERATED CHELAE WITH RESPECT TO LENGTH OF PROPUS, DACTYLUS, AND MERUS: RELATIVE THICKNESS OF CHITIN: LENGTH OF INTERSEGMENTAL SEPTA: AND PINCER DEVELOPMENT

	Propal Length up to 2.0 mm.							Propal Length longer than 2.1 mm.						
	Number of claws	Propus	Dactylus	Merus	Chitin thickness	Length of Septa	Development of pincers	Number of claws	Propus	Dactylus	Merus	Chitin thickness	Length of Septa	Development of pincers
Experiment 2 (12 days)														
Control	3	17.0	11.5	3.0	2.0	3.0	1.7	6	25.0	17.5	9.3	2.2	3.2	2.7
M/50,000	4	18.0	12.5	3.0	3.2	3.7	2.0	3	22.3	16.0	6.3	3.7	7.7	10.0
M/100,000	6	15.4	8.7	4.4	3.8	4.3	2.3	2	23.0	16.5	7.0	4.0	8.0	10.0
Experiment 3 (11 days)														
Control	3	11.0	9.0	4.2	2.7	5.0	3.7	4	23.2	15.2	5.2	1.5	4.7	1.2
M/50,000	2	19.0	11.0	3.0	1.5	3.0	2.0	1	26.0	20.0	9.0	3.0	5.0	1.0
M/100,000	3	14.3	8.7	6.3	3.0	3.7	2.0	4	24.7	19.0	8.5	3.5	6.0	9.0
Experiment 4 (11 days)														
Control	5	17.0	11.4	4.8	3.2	3.5	1.8	1	22.0	16.0	9.0	2.0	4.0	3.0
M/50,000	2	17.0	9.5	5.0	3.5	4.5	5.0	4	28.5	22.7	12.2	2.7	5.5	3.2
M/100,000	6	16.0	9.3	5.0	3.0	4.7	1.2	4	24.2	18.0	6.5	2.5	6.5	7.7
Experiment 5 (11 days)														
Control	4	17.7	11.3	4.7	2.0	4.7	2.2	2	22.0	14.5	6.0	3.5	4.5	1.5
M/50,000	6	16.1	11.5	4.1	3.3	4.5	5.0	2	22.5	15.0	7.0	4.0	5.0	2.0
M/100,000	7	16.4	12.1	4.7	2.9	4.7	5.3	2	23.5	21.0	6.5	3.0	8.0	10.0
Averages of All Chelae														
Control	15	15.8	10.8	4.4	2.5	4.1	2.3	13	23.9	16.2	7.5	2.2	3.9	2.1
M/50,000	14	17.2	11.4	4.3	3.1	4.1	3.7	10	25.6	18.9	9.1	3.3	6.0	4.8
M/100,000	22	15.7	10.0	4.7	3.2	4.5	2.9	12	24.1	18.6	7.2	3.2	6.8	8.9
Averages of Experiments 2, 4, and 5														
Control	12	17.2	11.4	4.4	2.5	3.8	1.9	9	24.0	16.7	8.6	2.4	3.6	2.4
M/50,000	12	16.9	11.5	4.5	3.3	4.3	4.0	9	25.6	18.8	9.1	3.3	6.1	5.2
M/100,000	19	15.9	10.3	4.7	3.2	4.6	3.1	8	23.8	18.4	6.6	3.0	7.3	8.9

Classification of the intersegmental septa presented no difficulties. The short with value 2 extended about a third across the chela. The long with value 4 extended two-thirds. When the septa went clear across they were marked 5. When articular differentiation was evident on one side, a value of 6 was given: and when this was on both sides and around the claw, 8 was granted.

Pincer development was similarly evaluated. Septal anlage was rated at 1; beginning and short septal chitin at 2; septam half along 3; two-thirds along 4; all along 5; partial articulation 8; full articulation and some denture 10.

Owing to a misunderstanding the chelae of experiment 1 were thrown away after notes had been made of their developmental status. The mathematical analysis therefore does not include these castaways. The analysis is based on differences of ten per cent or over.

RESULTS

Chela size is indicated by the over-all and individual length measurements of the constituent segments. From the averages of all chelae as given in Table II it is seen that chela size was about the same in the l-proline series as in the controls within the ten per cent limit set for comparison. One apparent exception occurs in the case of the M/100,000; 2.1 mm. and over set. This is due to over-weighting values from experiment 3. The regenerates in this experiment had a hard time and many were lost during removal. The values recorded may therefore not be representative. Assuming they are not and calculating the averages from experiments 2, 4, and 5, it is seen that in no case did test and control chelae differ significantly with respect to size.

Breaking down the averages into their 16 components it is found that the l-proline chelae were larger than their controls in 6 instances; smaller in 2: and equal in 8 with no consistency as to distribution. Leaving out experiment 3, it is seen that in the remaining 12 components the l-proline chelae were larger than their controls in 2 instances; smaller in 2; and equal in 8. Thus by consistency as by averages it is evident that l-proline produced no regular size differences in this material.

Turning now to developmental differences it is seen from the averages that despite the fact the claws were no larger the chitin was thicker, the intersegmental septa longer, and the pincer septal separation more advanced in the l-proline than the control series throughout. This holds in the separate experiments and the divisions thereof as well Thus in the 16 components, test development was more than control in 12; less in 2; and equal in 2. Omitting experiment 3, the numbers are 11, 0, and 1 respectively. The betterment is not confined to any one expression as far as incidence is concerned; but it is essentially the same for chitin, septa, and pincer. The degree of increased structural differentiation seems to have been greater in the case of the pincer, than in the case of chitin or intersegmental septa. This is indicated in the averages and sustained in separate experiments with the exception of experiment 3. In the 12 remaining components pincer development exceeded that of chitin or septa in 8 instances and was less in but 4.

Representative examples of the differentiation reaction to l-proline are given in the accompanying photographs. Figs. 1, 2 and 3 are of claws in which the Propus was 2.0 mm. or less in length: Figs. 4 and 5 from claws longer than 2.1 mm. Tests and controls are strictly comparable with respect to experiment, concentration, and length of Propus.

DISCUSSION

The measurements demonstrate and the pictures visualize the fact that l-proline acts to forward structural differentiation in the regenerating hermit crab chela beyond peradventure. The thickened chitin, the intersegmental septal extensions and unfoldings, and the development of pincer articulation and denture which took place in the presence of this amino acid well beyond the degree expressed in control chelae *of equal size* are unquestionable evidences. To those of us who were familiar with this material, the extent of pincer differentiation was most striking, for we had never encountered anything like it in previous experiments, even when they were run for longer periods under much more favorable conditions.

Perhaps it may be well to use a little space to restate the basis of the decision. The growth in size of the regenerating chelae during the period of observation obtaining in these experiments is mainly factored by cell increase in number (Hammett and Hammett, 1932). Other things being equal, chelae of like size are expressions of like increases in cell number. Therefore since the chelae in the l-proline cultures were not appreciably larger than their controls it can be concluded that the amino acid did not act to forward cell increase in number in this material. The marks of differentiation expression in the chelae are chitin deposition, separation and articulation of the segments by chitinous septa, and separation and articulation of the two parts of the pincer. Therefore when these occur to greater degree in test chelae of the same size as control, as they did in these experiments, the only conclusion possible is that the experimental procedures produced a specific forwarding of differentiation expression.

It should be stated that although no measurements were made of the chelae of experiment 1, the observations made thereof under the binocular were consistent with the findings reported in preceding paragraphs.

Consistent and sustaining of these is the finding by Wilson (1940) that l-proline conditions a like acceleration of structural differentiation in Drosophila larvae; the finding of Miller and Reimann (1940) of like effect on rabbit ova cultured in vitro; and the report of Hammett (1940) that spontaneous mouse tumors are similarly affected.

All these data extend the observations made by Hammett and Collings (1937) with Obelia to other and most diverse species. They

show the influence is on the same basic growth activity whether the organism is a coelenterate, a crustacean, an insect, or a mammal. And because of this, they allow the conclusion to be made that this naturally occurring amino acid of general distribution provides a chemical group which is specifically significant to some process essential for differentiation expression. It is probable that it is not proline as such but some characteristic derivative thereof—such as pyrrolidone—which is the effective agent. The evidence for this decision is presented elsewhere (Hammett, 1938).

Now since the influence is not species, organo, or cyto-specific, but is specific only to differentiation as a general aspect of developmental growth, it is germane to present interpretation to attempt closer definition. This can perhaps best be done by a somewhat free quotation from Section IV of The Nature of Growth (Hammett, 1936).

The factors which determine that a new cell shall differ from the old are distinct from the factors which determine what the difference shall be. The distinguishment is between the *potentiality for* and the *nature of* the change: between the *capacity for* and the *quality of* differentiation.

Though the end products of the developmental growth activity of differentiation are interminably diverse, potentiality for the expression thereof is at one time or another common property of living matter wherever found. That is to say, there is a single chemical reaction basis for differentiation. This is composed of those definite and specific chemical groups and/or arrangements essential for the expression of the activity as a fundamental property of growing things. It is a reaction basis which is different from those which determine specificity of cell type and composition.

Cellular and its derived structural differentiation is an expression of chemical specialization. Growth by differentiation—like all other growth—is a becoming, not a sudden acquisition. To its accomplishment contribute at least two sets of general processes—the enabling and the expressive.

The expressive factors produce the cellular composition peculiar to the species, the organ, and the structure. These are set by genetically specific factors. The enabling factors unlock the composition of the mother cell so that the potentiality of the daughter cell for differentiation may be expressed. They set into action those inter- and intramolecular rearrangements which take place at the redistribution and resynthesis stage of cell division wherefrom the potentiality for being different according to the genetic constitution comes to expression. The enabling factors are therefore assumedly common to living substance wherever found.

From the foregoing and from the fact that l-proline forwards differentiation not of a particular cell, but of diverse cells it can be postulated that the amino acid or some naturally occuring derivative thereof is an *enabling* factor for this growth activity.

SUMMARY AND CONCLUSION

Five experiments were run in which regeneration of the right chela of *Pagurus longicarpus* took place in control sea-water cultures and sea-water plus l-proline in M/50,000 and M/100,000 concentrations.

The structural differentiation of the regenerating member was enhanced by the amino acid despite the absence of any increase in size. This was expressed by a greater chitin deposition in test structures of equal size with controls; a more extensive development of intersegmental septa; and a more advanced development of the pincer, even up to the stage of articulation and denture. Comparative photographs are given. Since this influence has been demonstrated in a wide variety of other organisms as well as in Pagurus it is concluded that the naturally occurring amino acid of general distribution—l-proline—is an enabling factor specific for differentiation.

It is probable that it is not l-proline as such, but its pyrrolidone derivative or some part thereof which is the effective agent.

The photographs were made by Lee Walp and Narcissa Williamson to whom we are grateful for their efforts.

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FURTHER LOCALIZATION OF SH-ACTION ON CELL MULTIPLICATION

By

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INTRODUCTION

It has been established that SH is a naturally occurring chemical group essential for cell increase in number (Hammett, 1929). Since early studies showed SH to be concentrated in the nucleus (Hammett, 1929a, and Hammett and Justice, 1929), and that it is nuclear rather than cytoplasmic division which responds most consistently to the chemical group (Voegtlin and Chalkley, 1930), it was suggested that the locus of its action is the nucleus (Hammett, 1935). But it was later found that *non-nucleated* cells, such as the blue-green algae, are also stimulated to multiply by SH (Hammett and Walp, 1939). It is therefore evident that the point of influence is not confined to any structure or process specific to division of a formed nucleus, but is related to some process of more basic and universal concern to proliferation.

For one cell to become two it is necessary that anatomical division take place. This division is not accomplished by cutting as by a knife; nor is it brought about by tearing as by autotomy. Rather does it occur by an endogenously determined separation of cytoplasmic and nuclear substance in a definite plane of cleavage.

It can be assumed that actual severance is preceded by a loosening of bonds between the protein molecules or of bonds uniting the amino acids in the protein molecules. That is to say, a partial proteolysis of cell substance along a definite plane of future cleavage is a chemical preliminary to cleavage itself.

Granting this and reasoning from general knowledge of protein behavior, it can be inferred that this particular phase of cell division is effected by a specific type of proteolytic enzyme. A type which does not 'digest' the protein by splitting it down to amino acids, or peptids, or peptones, or even proteoses, but merely acts to loosen bonds with little or no loss of cellular protein integrity. At least when its action is exerted under the regulatory conditions of living systems.

Enzymes which apparently have such restricted action are known and are called proteinases. These have the remarkable property of being activated by SH. In fact, it is generally assumed that SH is the

naturally occurring activator of this class of enzymes (Waldschm t-Leitz et. al., 1933; Grassmann et. al., 1931; Maschmann and Heln t, 1933).

Correlating these observations, the postulate has been advanced y Hammett (1934) that cell increase in number is forwarded by H because this group is the activator of the proteinase responsible for at partial proteolysis of cellular protein which necessarily prec es separation of daughter from mother cell.

Direct experimental verification of the postulate has yet to be m le. A study of the relative proteolysis of cytoplasmic and nucleo-prot ns exposed to proteinase might be a useful step in this direction. Such as been attempted here.

MATERIAL AND METHOD

Protein which is indisputably cytoplasmic is essentially unavailable. Therefore the storage protein edestin, and the secretion protein egg albumen were used as representative of non-nuclear proteins. Representative of nucleo-proteins were a protamine kindly supplied by Eli Lilly & Company through the courtesy of Dr. George H. Clowes; a liver nucleo-protein generously given from the slender store of Dr. Jesse P. Greenstein of The National Cancer Institute; and a beta-nucleo-protein prepared from hog pancreas by Dr. Lavine according to Steudel and Brigl (1910).

The egg albumen contained 3.50% H_2O and 14.67% N; edestin, 7.15% H_2O, 16.88% N; and the B-nucleo-protein, 4.27% H_2O, 12.39% N, 5.79% P. Optimo Papain (S. B. Penick & Company) was used as the enzyme. It contained 4.23% H_2O and 8.00% N.

The proteinase solution was freshly prepared for each experiment by grinding 0.250 gm. papain in a mortar with a little water. This was let stand for about 1½ hours with occasional stirring (while the protein solutions were being prepared) and then filtered into a 50 ml. volumetric flask. Ten ml. of this slightly yellow solution were used for each gram of protein in the hydrolyses.

A weight of glutathione equal to the weight of papain powder was used for activation of the enzyme.

The concentration of protein in solution or in suspension was 1 gm per 100 ml. Protamine and B-nucleo-protein were soluble. Egg albumen, edestin and liver nucleo-protein were in suspension. The insoluble proteins were ground in a mortar with a little distilled water to make a fine suspension. They were then transferred to a 50 or 25 ml. volumetric flask and the requisite amount of 0.2 M sodium citrate buffer added so that on dilution to volume the mixture was 0.06 M.

The pH of the mixture was 5. It did not change appreciably during hydrolysis. After adding the enzyme (and glutathione when used) to the buffered mixture, the contents of the flasks were diluted to volume and shaken to insure homogeneity.

An aliquot was taken and filtered for the Sorensen formol titration (Mitchell and Hamilton, 1929). A second aliquot was added to an equal volume of 10% CCl_3COOH and amino nitrogen was determined in the filtrate by The Van Slyke manometric procedure (Peters and Van Slyke, 1932). These gave the N values prior to hydrolysis.

The enzyme-protein mixture was then allowed to hydrolyze at 38±1°C. After the required period, the flasks were cooled to room temperature and aliquots analyzed as noted above. The formol titrations were always performed at once, usually with aliquots of 4 cc; while the solutions with CCl_3COOH could stand for at least a day without change in NH_2-N value.

In the formol titration, a 4 ml. aliquot of the protein solution was brought to pH 7 by the addition of 0.2 ml. of N NaOH using neutral red and a reference standard. A volume of 36% formaldehyde equal to 20% of the total final volume was then added and titration completed to pH 9 using thymol blue as indicator and a reference standard. Preliminary titrations with the amino acids leucine and valine showed that a formaldehyde blank was necessary for quantitative results. This varied with different lots of formaldehyde. The results shown have been so corrected. The deviations with buffered protein solutions were greater than with pure amino acids and amounted to ±0.0001 milli-atoms of NH_2-N per ml. of protein solution.

For the Van Slyke determination of amino nitrogen 5 ml. of the trichloroacetic acid filterate were used in the case of egg albumen and edestin; 3 ml. in the case of protamine; and 2 ml. in the case of B-nucleo-protein and liver nucleo-protein. The results obtained on the CCl_3COOH filtrate from the protamine solution are perhaps somewhat in error since protamine itself yields a considerable amount of NH_2-N, which is not precipitable by CCl_3COOH after digestion. This is best illustrated by the following figures. The protamine solution contained 0.0089 milli-atoms of NH_2-N before hydrolysis and 0.0107 milli-atoms after 24 hours hydrolysis with activated papain, or an increase of 0.0018 milli-atoms of NH_2-N per ml. The same determinations of NH_2-N in CCl_3COOH filtrates are 0.0062 milli-atoms before and 0.0109 milli-atoms after hydrolysis or an increase of 0.0047 milli-atoms of NH_2-N. There is only a very slight precipitate produced by the trichloroacetic acid even after 1½ hours hydrolysis of protamine. This means that the protamine results in Table I are all too high.

However a correction would only accentuate the difference between protamine and egg albumen and edestin and not change the direction of the difference. Only a slight precipitate is produced by CCl_3COOH with B-nucleo-protein at any time.

TABLE I

THE INCREASE IN THE AMINO-ACID NITROGEN OF CYTOPLASMIC AND NUCLEO-PROTEINS AFTER 24 HOURS ACTION OF GLUTATHIONE-ACTIVATED PROTEINASE (PAPAIN)
AMINO NITROGEN IN MILLI-ATOMS PER LITER

	Cytoplasmic Proteins						Protamine			Nucleo-Proteins					
	Egg Albumen			Edestin						Beta-Nucleo-protein			Liver Nucleo-protein		
	Beginning	End	Increase	Beginning	End	Increase	Beginning	End	Increase	Beginning	End	Increase	Beginning	End	Increase
	N_2H—N by Van Slyke Procedure														
1	4.6	11.1	6.5	4.6	9.9	5.3	7.7	11.7	4.0	15.3	19.8	4.5	5.0	20.3	15.3
2	4.6	12.1	7.5	4.6	10.5	5.9	7.4	11.5	4.1	15.3	19.7	4.4	4.9	15.9	11.0
3	4.8	9.9	5.1	5.7	10.6	4.9									
4	4.6	9.9	5.3	4.5	10.1	5.6									
5	4.6	9.8	5.2												
6	4.9	9.8	4.9												
Av.	4.7	10.4	5.8	4.9	10.3	5.4	7.5	11.6	4.0	15.3	19.7	4.4	4.9	18.1	13.1
	N_2H—N by Formol Titration														
1	3.9	9.4	5.5	3.8	9.4	5.6	9.7	10.7	1.0	18.2	19.5	1.3	11.2	23.9	12.7
2	5.1	9.4	4.3	5.1	10.0	4.9	9.5	11.4	1.9	17.5	18.9	1.4			
3	5.7	10.0	4.3	5.4	10.3	4.9									
4	5.1	9.8	4.7	5.2	10.4	5.2									
5	5.4	9.4	4.0	4.4	8.5	4.1									
Av.	5.0	9.6	4.6	4.8	9.7	4.9	9.6	11.1	1.4	17.8	19.2	1.3	11.2	23.9	12.7

Each NH_2-N result listed is the average of two determinations which checked within ± 0.0001 milli-atoms per ml.

In order to insure uniformity of conditions, egg albumen and edestin were run simultaneously with each of the several nucleo-proteins.

RESULTS

The effect of activated proteinase on the various proteins are shown in Table I. The data reveal that the extent of hydrolysis over a 24 hour period was less with protamine or B-nucleo-protein than with either egg albumen or edestin. Liver nucleo-protein on the other hand was hydrolyzed to a greater degree than any of the other proteins and in fact (with respect to both solubility and hydrolysis) behaved more like egg albumen or edestin than like protamine or B-nucleo-protein.

In order to determine whether the papain had exerted its maximum hydrolytic effect on protamine or B-nucleo-protein in 24 hours, which was evidently not the case for egg albumen, edestin or liver nucleo-protein in view of the insoluble protein remaining, a preliminary inves-

tigation was made at shorter intervals. The results are given in Table II which shows the extent of hydrolysis after 1½ and 3 hours as measured by the Van Slyke procedure. The 24 hour figures are the maximum values of Tables I and III.

The data indicate that although protamine and B-nucleo-protein are hydrolyzed to lesser degree than either egg albumen or edestin, they are on the other hand more rapidly attacked by the enzyme.

Just to check up on our material a comparison of the proteolytic effect of SH-activated papain with that of the unactivated enzyme was made over a 24 hour period. As usual, the NH_2-N was measured by the Van Slyke procedure. The results are given in Table III. The higher 'beginning' values in the SH-activated series are of course due to the added glutathione. This is shown by the fact that the difference between these and the 'beginning' values of the unactivated series without added glutathione is of the same order of magnitude for all examples.

As should have occurred in view of the reports in the literature, the addition of SH to the protein-enzyme mixture enhanced proteolysis. There was one exception, viz. protamine. This exception will be discussed in a later section.

TABLE II

THE AMINO-ACID NITROGEN PRODUCED BY GLUTATHIONE-ACTIVATED PROTEINASE (PAPAIN) IN 1½, 3, AND 24 HOURS

	Amino nitrogen in milli-atoms per liter			In percents of 24 hour yield	
Protein	After 1½ hours	After 3 hours	After 24 hours	After 1½ hours	After 3 hours
Protamine	5.0	5.1	4.9	102	104
Beta-Nucleo-Protein	2.0	3.1	4.5	44	69
Edestin	1.2	1.8	5.9	20	31
Egg Albumen	1.2	2.1	7.6	16	28
Liver Nucleo-protein	2.2	2.8	15.3	14	18

DISCUSSION

Understanding of biological phenomena progresses as pertinent observations from diverse disciplines are brought together and integrated into interpretation. The conceptual extension which emerges from such correlation is obviously merely an approximation. However all concepts are but approximations. And it is not until all pertinent disciplines are used in the attack upon a given problem that a concept can be arrived at which truly evaluates the process-as-a-whole.

The present study is an attempt to dig further into the site and mode of action of SH in cell division. It starts from certain known facts derived from the diverse disciplines of bio-chemistry and cytology, and goes on from there to experimental testing of their possible relation using certain assumptions which are at present unavoidable. These will be explicitly noted as the argument proceeds.

TABLE III

COMPARISON OF PROTEOLYTIC ACTIVITY OF GLUTATHIONE-ACTIVATED PROTEINASE (PAPAIN) WITH THAT OF THE UNACTIVATED ENZYME. NUMBERS ARE MILLI-ATOMS OF AMINO-NITROGEN PER LITER IN 24 HOURS

Unactivated			Activated			
Start	After 24 hours	Set free by enzyme	Start	After 24 hours	Set free by enzyme	Percentage increase by Activation
			Egg Albumen			
1.1	3.5	2.4	4.6	10.3	5.7	138
1.1	4.3	3.2	4.7	10.1	5.4	69
1.0	4.3	3.3	4.6	9.7	5.1	55
1.0	4.4	3.4	4.7	9.5	4.8	41
			Edestin			
1.0	2.2	1.2	4.4	7.5	3.1	158
			Protamine			
1.7	5.8	4.1	5.7	10.3	4.6	12
1.8	6.8	5.0	6.1	11.0	4.9	— 2
			Beta-Nucleo-protein			
11.7	12.7	1.0	15.5	19.5	4.0	300
12.1	12.6	0.5	15.9	19.2	3.3	560
11.9	12.8	0.9	15.5	19.3	3.8	322
			Liver Nucleo-protein			
1.3	11.4	10.1	5.0	20.3	15.3	51
1.1	9.3	8.2	4.9	15.9	11.0	34

The living substance of the cell is largely protein in nature. There are three general types of protein in the cell; the extra-nuclear or cytoplasmic protein, and the nuclear proteins which are two, viz. that comprising the nucleoplasm, and that of which the chromatin is made. The evidence of distinction between nucleoplasmic and chromatin proteins is mainly cytological. Biochemists have yet to set them apart in clear-cut separation. It is probable that nucleoplasmic protein approximates that of cytoplasm rather than that of chromatin in composition.

The egg albumen and edestin used in this study may or may not be true examples of cytoplasmic protein. They will however be assumed so to be since their amino acid makeup approximates the analysis of non-nuclear proteins (Mitchell and Hamilton, 1929). They are distinct from that of the protein moiety of the chromatin protein which is largely composed of basic amino acids. The beta-nucleo-protein may be assumed to be representative of chromatin protein for it is composed of a histone-like protein conjugated with nucleic acid (Jones, 1920). The protamine is representative of the protein moiety of chromatin. It is largely composed of basic amino acids and is not conjugated with nucleic acid (Matthews, 1931). The liver nucleo-protein, so kindly furnished by Dr. Greenstein, seems to be more representative of a nucleoplasmic than of a chromatin protein. The evidence for this assumption is that it was more like egg albumen and edestin in solubility and hydrolytic behavior than it was like protamine or beta-nucleo-protein, and its phosphorus content was below that ordinarily associated with the conjugated protein of chromatin (Greenstein and Jenrette, 1940). It is to be remembered that the cytoplasmic (sic) proteins were essentially insoluble while the nuclear products (protamine and beta-nucleo-protein) were soluble. It is possible that the liver product was largely nucleoplasmic protein with some slight admixture of chromatin protein.

With these distinctions set forth it becomes possible to begin exposition of the results given in the preceding section.

The data of Table I show that both cytoplasmic and nuclear proteins are hydrolyzed by SH-activated proteinase, and the data of Table III show that SH enhances the process. This is taken to mean that SH participation in cell division insofar as proteolysis is concerned, is not confined to the nucleus as was at first supposed, but extends to the cytoplasm. It also means, as might have been expected, that both nuclear and cytoplasmic protein have similar bonds between the constituent molecules or between the amino acids which comprise them. It therefore sustains the previously expressed hypothesis that SH action in increasing cell proliferation is on a process common to division of the cell as a whole rather than on a process restricted to one structural component of the cell as distinct from another.

The data of Table I show that the cytoplasmic proteins undergo more hydrolysis in 24 hours than do the chromatin products. This is taken to mean that there are more bonds of the particular type whose loosening is a preliminary to separation of daughter from mother cell in the cytoplasm than in the chromatin component of the nucleus. The inference is that chromatin protein is more stable to this type of proteolysis than is cytoplasmic. This finding is consistent with the probability

that cytoplasmic activity in general is greater than that of the chromatin moiety of the resting nucleus, a belief which derives from obsertions on nitrogen balance, inanition, and the recent reports that a continuous interchange of amino acid nitrogen takes place as a normal accompaniment of living.

In order to supplement these *in vitro* studies, one of us (F. S. H.) investigated the action of SH-activated proteinase on living cells using the same mixtures as were employed in the protein studies. Free hand sections were made of young and tender root-tips of grass. One set was immersed in proteinase solution and one in the medium without proteinase. The nuclear-cytoplasmic status was examined before immersion, and after immersion for varying periods. It was generally found that the cytoplasm gave evidence of early dissolution while the nucleus was more resistant. Though the nucleus also gave evidence or disturbance after prolonged exposure, it persisted as a unit structure, whereas the cytoplasm was well dispersed. These observations then are consistent with the others and sustain the implication that nucleo-protein is less subject to the proteolytic action of proteinase than is cytoplasmic.

Such a finding is consistent with the belief that chromatin is the bearer of those chemical groups whereby heredity is determined. For here if in no other substance one would demand stability; otherwise persistence and continuity of hereditary characteristics would be impossible.

Table I shows it is quite otherwise with liver nucleo-protein. This material hydrolyzed to much greater extent than did the chromatin-like products. This means that this protein had many more bonds capable of being loosened by proteinase than did the chromatin proteins. This takes it immediately from that class and groups it with egg albumen and edestin. In other words, the product can be assumed to represent a nucleoplasmic rather than a chromatin-like protein.

This nucleoplasmic preparation hydrolyzed to greater extent than did the other plasmic products. This may be due to the probability that it is a more representative derivative of active cytoplasm, or it may be an expression of an innately greater activity. There is a belief, which has some experimental foundation, that nucleoplasmic activity is the governor of cytoplasmic expression. If this be so, it might follow that nucleoplasm is more labile than cytoplasm insofar as nitrogen or amino acid interchange is concerned and hence is naturally more sensitive to partial proteolysis. Such sensitivity would be a necessary requisite for lability otherwise the continuous interchanges necessary for activity expression could not be had. The data are at least suggestive in their consistency.

The data of Table II show that hydrolysis of the chromatin proteins takes place much more rapidly than that of the plasmic. In fact that of protamine is completed within an hour and a half. This is taken to mean that the essential loosening of the bonds occurs earlier in chromatin than in plasm. This is consistent with the sequence of events in karyokinesis. The chromosomes do split and separate before the plasmic mass of the one cell is separated into the two.

It may be that solubility was the basis of the early completion of proteolysis in protamine and beta-nucleo-protein. Such a conclusion, however, would not necessarily invalidate the comparison with early chromosome splitting, for it could *pari passu* be inferred that this too is factored by differential solubility.

Liver nucleo-protein follows egg albumen and edestin rather than protamine and beta-nucleo-protein with respect to percentage hydrolysis during the early stages. This finding again groups it with the plasmic rather than the chromatin proteins, and supports the assumption it is a representative of nucleoplasm. This assumption is not negatived by the fact that the enhancement of proteolysis produced by SH is by and large of the same order of magnitude in the liver product as it is in egg albumen.

It may be noted here that the hydrolytic data so far produced separate the proteins into two groups without overlapping as do their origins.

The comparative influence of SH on proteolysis is of particular interest. This is factored primarily by the amino acid composition. Solubility is of lesser importance since, as shown by the data of Table III, the two soluble proteins protamine and beta-nucleo-protein differ widely in extent of reaction.

Protamine, of all the proteins studied, was essentially unaffected. This means that, within the limits of the experimental procedure, SH is of limited influence on the partial proteolysis of this chromatin component. It may be assumed then that this protein moiety of chromatin is a most stable type of cellular proteins. But this is just what might have been expected since it is in this particular type of cellular component that the chief chemical factors for heredity are presumed to reside. The conclusion is based on the assumption that genes are carried by the chromosomes which are composed of chromatin; that they are composed of amino acids in specifically coordinated groups and combinations; and that for the exercise of heredity they must be essentially indissoluble. Admittedly this is a speculative interpretation and correlation. Despite this, the possibility is worth calling attention to.

The large increase in proteolysis of beta-nucleo-protein by SH is also significant. The compound is a conjugated protein in which nucleic acid is a characterising component. None of the other proteins are similarly composed. It therefore may be assumed until other studies disprove it that the great effect of SH on beta-nucleo-protein hydrolysis is conditioned by the presence of nucleic acid in the molecule.

If it be granted that the beta-nucleo-protein used in these studies is fairly representative of the chromosomes because it is chemically similar thereto by virtue of its being a compound in which basic protein is conjugated with nucleic acid, then the fact that its proteolysis is markedly enhanced by SH may mean that SH is of specific importance to that partial proteolysis of the chromosomes which is an essential preliminary to their division. Supporting this is the finding reported earlier that SH acts to increase chromosome thickness (Hammett, 1934).

The fact that the hydrolysis of protamine, another known constituent of chromatin, is essentially unaffected by SH, while that of beta-nucleo-protein is affected, not only provides a distinction which tends to justify the allocation of SH action to chromosome splitting; but it also provides a basis for interpretative correlation of the phenomenon with cytologic and genetic observation. These teach that during cell division the material of which the chromosomes are composed is separated into two portions and that this separation of the material does not destroy its chemical integrity.

Now it is obvious that the chromatin proteins which are analagous to beta-nucleo-protein undergo partial hydrolysis and that SH is a naturally occurring stimulus therefor. This makes possible the division of the chromosomes, the determining factor for which is unknown. This partial proteolysis, however, does not destroy the integrity of the basic substance of the chromosomes as is attested to by cytologic and genetic studies. It therefore may be postulated that from this preliminary partial proteolysis result proteins which are essentially resistant to further dissolution even in the presence of the stimulating influence of SH. The experiments of this report are consistent and sustaining of this postulate since they show that protamine, a simple unconjugated protein component of chromatin, exhibits these necessary characteristics.

These relations then clearly justify the allocation of SH action on the chromosomes to the stage of their division. They also indicate there is close correlation between chemical and chromosomal changes from which interpretation of the latter in terms of the former may be had.

SUMMARY AND CONCLUSION

Proteins representative of nuclear and cytoplasmic substance have been subjected to partial proteolysis by proteinase obtained from papain.

It was found as has been reported by others that the addition of SH enhances the hydrolytic action of the enzyme.

It was found that the plasmic proteins, both cyto- and nucleo-, were hydrolyzed by SH-activated proteinase to greater extent than the chromatin-associated proteins. This may be expression of the relative extent to which the two types participate in the activities of living.

It was found that the chromatin-associated proteins tended to complete their hydrolysis in the presence of SH-activated proteinase earlier than the plasmic. This is correlatable with the fact that chromosome division precedes cell division.

It was found that SH-activated proteinase caused partial proteolysis of both cytoplasmic and nuclear proteins.

The conclusions are that the increased cell multiplication produced by SH derives in large part from the stimulating effect of this naturally occurring chemical group on that partial proteolysis which is the essential preliminary to the division of one cell into two; and that the influence is not confined to any particular structure, but is exerted on both nuclear and non-nuclear division.

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A SUGGESTED MECHANISM FOR THE REGULATION OF PROPORTIONATE GROWTH, SUPPORTED BY QUANTITATIVE DATA ON THE BLOOD NUTRIENTS[1]

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Aside from the basic nature of protoplasmic synthesis itself, possibly the most important problem of growth is the manner in which the component parts of the developing organism achieve and maintain their characteristic size relationships. This is the problem of proportionate or relative growth. The method of embryonic transplantation has offered a fruitful approach to the analysis of this question, by permitting one to create disharmonious size relationships between organ and host and thereby to test for the operation of factors which may restore the normal balance.[2] The transplantation of organ rudiments between species differing markedly in their rate of growth has been particularly useful. Certain organs, such as the heart (Copenhaver, 1930, 1939) and ear (Richardson, 1932), adjust their size completely or in part to that appropriate for their hosts, apparently in response to functional requirements or mechanical limitations of space imposed by their new environment. Others, notably the eyes and limbs, (Twitty and Schwind, 1931), appear free to express their customary or genetic growth rate in relative freedom from such interference. Obviously, this does not mean that growth of the eyes and limbs is independent of regulation by the organic environment. One must infer instead that the factors which coordinate the growth of these organs with that of the rest of the animal are virtually identical in the two species used for the exchange and hence are not revealed by the experiment in question. That such factors exist, and exert an important control on organ growth, was demonstrated by exchanging eyes in larval stages between younger and older individuals (Twitty, 1930; Twitty and Elliott, 1934). Normal size relationships between organ and host, disturbed by this exchange, were restored through appropriate modification in the subsequent growth of the transplants.[3]

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[1]The investigation was supported in part by a grant from the Rockefeller Foundation.

[2]For reviews of the literature on this subject see Harrison, 1933; Twitty, 1934; and Twitty, in press.

[3]Bodenstein (1940) has recently obtained similar results following the transplantation of eye and limb discs between younger and older larvae of Drosophila.

Whatever the specific nature of the size regulating mechanism responsible for these results may be, it is evident that it involves progressive changes during larval growth in both the eye and its organic environment. Thus a given eye finds different conditions for growth on animals of different age, since it may be either accelerated or decelerated in growth, depending upon whether the host is older or younger than the graft. The response of the eye itself also changes with age, since a younger eye is stimulated, an older one retarded when grafted to a given host of intermediate age.

In an effort to construct a suitable hypothesis, it has been proposed that the changes in the eye itself consist of a gradual decline in the "assimilative capacity" of its constituent cells. This assumption is not without *a priori* foundation, since it is generally accepted that growth activity (as measured by percentage increase) tends to fall off as size advances. Particularly clear evidence for the existence of such a differential seems to be offered by the following experiment (Twitty and DeLanney, 1939). Eyes were grafted from younger to older larvae as before, but instead of feeding the hosts, they were starved from the time the transplantations were made or even earlier. Under these conditions the hosts and their normal eyes were of course unable to accomplish further growth. The young transplants, on the other hand, achieved a very significant increase in their size. The younger organs are thus capable of acquiring materials for growth under conditions which are subthreshold for their older mates.

This assimilative advantage associated with physiological youth is undoubtedly a factor in the regulative growth of organs grafted to older hosts, but it will not alone account for the observed facts. It explains why such a transplant grows faster than the normal organ of the host, which is of course essential if size regulation is to be accomplished. However, in order for the transplant fully to overcome its initial disadvantage in size, it must not only grow faster than its new mate, it must actually exceed its *own* normal growth rate. Or, stated differently, since it starts with an absolute handicap, it cannot overtake the normal eye of the host merely by growing more rapidly than the latter during the period of the experiment. It must set a faster pace than was achieved by the host eye even prior to the transplantation, that is, when the latter was itself the age of the transplant.

It was at first believed that the impetus to growth provided by the older hosts might be of an indirect nature, and could be interpreted by invoking the principle of competition in the partition of food stuffs between graft and host (Twitty, 1934). Granting a decline in the assimilative capacities of the tissues during larval growth, an organ grafted to an older host would be placed in competition for available

nutrients with cells less "avid" than those constituting the donor animal. This would give the transplant a competitive advantage which it lacked in its original environment, and account for the accelerated growth essential to complete size regulation.

However, subsequent experiments undertaken to test this hypothesis tend to minimize, if not discredit, the importance of competitive interplay as a factor in food partition. In demonstrating this, advantage was taken of the fact that regenerating organs follow the same rules as organs grafted to older hosts. After amputation of the limbs or tail, regeneration buds or blastemata form which appear to be physiologically much younger than the tissues of the rest of the animal. In fact, experiments have shown that they share certain properties of embryonic rudiments (Schotté, 1939). They grow actively, even under starvation, and eventually tend to restore the organs to their normal size. Since the tail is a relatively large organ, and utilizes corresponding quantities of food in the course of its regeneration, it was considered possible that regeneration of this organ might limit the amount of nutrients available for size-regulation in young transplanted eyes. However, this did not prove to be the case. The growth of such transplants was as great on hosts undergoing regeneration of the tail as on hosts in which the latter organ was intact (Twitty and DeLanney, 1939). Both series of animals were subjected to complete starvation during the experiment, and if competitive restriction does not manifest itself under these adverse circumstances, it seems unlikely that it would be an important factor under ordinary conditions of growth when the supply of food is not limited. "We must, of course, assume that competition . . . . . is potentially inherent in any closed system such as the starved organism, but evidently it is not likely to become operative. . . . . there is at least a moderate abundance of nutritive materials in circulation even under starvation. These are gradually depleted, but apparently not to the point where competition becomes a vital factor in their distribution." (Twitty and DeLanney, p. 411).

Since it appears that the postulate of competitive interplay must be abandoned in the interpretation of our results, it becomes necessary to seek another basis for the stimulating action of older hosts on the growth of organs grafted from younger donors. That the opportunities for growth are indeed superior in older as compared with younger larvae seems particularly clear from reinspection of one of the experiments already cited. When an eye is grafted to an older host, and the latter is subsequently starved, the ability of the transplant to grow while the host remains static or even diminishes slightly in size was offered as evidence that the tissues of the host possess a lower capacity to acquire food at a given level of availability than those of younger

larvae. The same experiment reveals another important differential, however, when we compare the performance of the transplant, not with that of its normal host organ as before, but with that of its original mate on the donor larva. Assuming that the latter animal, like the host, is starved after yielding the transplant, it and its remaining eye would have of course accomplished no further growth. The conditions for growth afforded a given eye are thus clearly more favorable on the older of the two larvae. (In fact it is presumably this superiority of the older environment which permits the transplant to express its higher assimilative capacities compared with those of the host tissues.) The same conclusion can be drawn from experiments in which the larvae are fed, through the more rapid growth of the graft compared with its donor control, but when only the former accomplishes any growth whatever, as under the conditions of starvation imposed here, the difference between the two environments is revealed even more concretely.

Perhaps the simplest postulate which one might make concerning the nature of the improvement in opportunities for growth during larval development is that it consists of a gradual rise in the level of critical nutrients carried by the blood stream (Twitty and DeLanney, 1939). The present investigation was undertaken to test this possibility by measuring the concentration of certain constituents of the blood at various stages of larval growth. The determinations were confined for the most part to total nitrogen, non-protein nitrogen and sugar. Amino acid nitrogen was also measured, but only in two instances.

MATERIAL AND METHODS

The larvae of *Amblystoma tigrinum* serving as a source of blood for the chemical determinations were grown in the laboratory from eggs collected near Nashville, Tennessee, and shipped by Dr. Karl Mason of Vanderbilt University. Tubifex worms and liver were used as food. The former were employed principally while the larvae were relatively small, and beef liver substituted later as their size and demands increased. However, as will be pointed out later, this procedure was modified in individual series to control the possible effect of type of food on the chemical results obtained. Food was withheld entirely for the last two or three days before blood was taken for analysis. Prior to this time, although no effort was made to insure maximal feeding, the larvae were all well fed for a period of at least several days. They were also maintained at essentially the same room temperature during this period.

In obtaining the blood samples, the larvae were placed on moist cotton after narcotizing with Basel Narcotic M. S. 222, and the heart completely exposed by cutting away the skin and floor of the pericardial cavity. The blood was drawn from the ventricle with a

micropipette attached to a rubber tubing held at the other end in the mouth. The pipettes were made by drawing larger glass tubing to a diameter of about 2 mm., and subsequently drawing the tip to a considerably finer bore (outside diameter roughly 0.4 mm.). The tip was further sharpened, under the dissecting microscope, by chipping with watchmakers' forceps until it tapered on one side to an acute point. Before each use these pipettes were flooded with potassium oxalate solution to reduce clotting of the blood. The thin heart wall offers insufficient resistance to permit ready puncture and must be held under tension with forceps while the pipette is being introduced. Lifting the heart in this manner also enables one to avoid drawing fluid of the pericardial cavity into the pipette. In order to prevent clotting and consequent difficulties in measuring and handling the blood, it was found necessary to pipette it immediately after withdrawal into a known volume (0.4 cc.) of oxalate solution. The same stock solution of potassium oxalate was employed throughout for this purpose. Several larvae, the number (from 7 to 25) depending upon their size, were required to provide a volume of blood adequate for analysis. In fact, larvae less than about 40 mm. in length yielded such small amounts that we have not as yet extended the comparisons into the early larval period. Even from groups of larger larvae the total volumes obtainable were at best small, about 0.2 to 0.4 cc. Thus, together with the oxalate solution (0.4 cc.), a total volume of about 0.6 cc. to 0.8 cc. was available for each set of determintions. After thorough mixing, such samples were divided into a sufficient number of portions, usually six, to permit two independent measurements of each of the constituents chosen for analysis.

Chemical determinations (W. J. van W.). Sugar was determined by the method of Hagedorn and Jensen (1922); total nitrogen, by the micro-Kjeldahl method after F. Pregl (1935); amino acid nitrogen, by the Van Slyke method (1932); and non-protein nitrogen by the micro-Kjeldahl method. For the amino acid nitrogen and the non-protein nitrogen determinations, the proteins were first precipitated. To the blood sample of 0.1 cc. was added 0.8 cc. distilled water and 0.1 cc. 2.5 n sulphuric acid (in a 15 cc. centrifuge tube). After standing for five minutes, the chocolate brown precipitate was centrifuged down and 0.8 cc. of the supernatant liquid was withdrawn by means of a pipette. In this sample the non-protein nitrogen was determined by the micro-Kjeldahl method. For the amino acid nitrogen determination 0.5 cc. of the supernatant liquid after centrifuging was used for each determination.

From the values obtained by these determinations, the amount in milligrams per cubic centimeter of blood was calculated (see Table).

EXPERIMENTAL RESULTS

The data thus obtained are presented in the accompanying Table. Briefly summarized, they present the following general features. (1) The concentration of total nitrogen and sugar remains essentially constant throughout the period of larval growth encompassed by the study. (2) The non-protein nitrogen, on the other hand, rises sharply with age. Since the total nitrogen remains constant, it follows that the concentration of protein nitrogen suffers a corresponding decline. (3) Although the non-protein nitrogen fraction presumably includes other forms of nitrogen as well, such as that represented in ammonium compounds and urea, the principal component is apparently amino acid nitrogen. This is evident from the high percentage of amino acid nitrogen in the two samples where it was measured directly.

It is true that the data, if plotted, would not present a smooth curve for the rise in non-protein nitrogen (note for example, the data for the 50 mm., 55 mm., and 56 mm. sequence of groups). Various factors, aside from experimental error, may account for these irregularities. For one thing, it was not possible to obtain larvae of completely uni-

TABLE I

MILLIGRAMS PER CUBIC CENTIMETER OF TOTAL NITROGEN, NON-PROTEIN NITROGEN, AMINO ACID NITROGEN, AND SUGAR IN WHOLE BLOOD DRAWN FROM LARVAE OF *Amblystoma tigrinum* OF VARIOUS SIZES

Avg. Length in mm.	Total N mg/cc		Non Protein N mg/cc		Amino acid N mg/cc		Sugar mg/cc	
		Avg.		Avg.		Avg.		Avg.
44	17.20	17.00	3.00	2.90			1.72	1.78
	16.80		2.80				1.84	
45	18.40	18.40	2.88	2.88			1.76	1.76
	—		—				1.76	
47	16.40	16.63	2.74	2.85			1.73	1.73
	16.85		2.96				1.73	
50	17.85	17.72	3.15	3.20			1.80	1.75
	17.40		3.26				1.70	
55	17.10	17.03	3.33	3.29	1.84	1.88		
	16.95		3.24		1.91			
56	16.80	16.97	4.41	4.62			1.50	1.56
	17.13		4.83				1.62	
70	16.40	16.40	6.76	6.76			1.40	1.40
	—		6.76				1.40	
72	16.35	16.35	11.76	11.45			1.73	1.73
	16.35		11.13				1.73	
85	16.90	16.80	11.20	11.30	9.54	9.34	1.76	1.76
	16.70		11.40		9.14		1.76	

form size for each set of determinations, with the result that there was frequently some overlapping in individual cases between successive groups. Moreover, because of technical difficulties, one does not obtain uniform volumes of blood from all of the larvae constituting a given group. Because of these two factors, it is possible that in some groups the average lengths as given do not provide an entirely accurate index of the "physiological age" of the blood samples. Our records suggest that this factor may partly explain the marked apparent difference in non-protein nitrogen content between the 70 mm. and 72 mm. groups. Another obvious factor which might influence the results is the possible effects of inadequately standardized level of feeding.

It was mentioned above that while the larvae were small they were fed principally Tubifex worms, followed by liver as they attained a larger size. Control series show that this difference in type of food does not affect the essential nature of our results. The 47 mm. group was fed liver for about ten days before they reached this size, whereas the larger 56 mm. group had been grown on worms until the time of their use. The results with these series are deemed sufficient to show that the higher percentage of non-protein nitrogen in the blood of larger larvae exists independently of mere type of food administered.

It is obvious that a much more exhaustive series of determinations must be made before a precise curve for the rise in non-protein nitrogen can be presented. The only conclusion which we wish to draw from the data now available is that there is apparently an unquestionable increase in the concentration of this fraction during larval growth, and that the rise involves principally an increase in amino acid nitrogen.

Before proceeding with the further discussion of these results, brief reference should be made to similar, although fragmentary, data on the blood of the slower growing *Amblystoma punctatum*. It was pointed out in the introduction that eyes and limbs exchanged between the two species reproduced their normal or donor rate of growth, which indicates that the larvae of both species afford about the same nutritive opportunities for organ growth. This suggestion appears to be supported by the data in question on the food content of *A. punctatum* blood. Only a single set of determinations were made, on a sample of blood taken from *A. punctatum* larvae averaging 38 mm. in length. The figures are as follows: total nitrogen, 17.03 mg./cc.; sugar, 1.70 mg./cc.; and non-protein nitrogen, 5.80 mg./cc. It will be noted from comparison with the Table that the values for total nitrogen and sugar correspond very closely to those which characterize *A. tigrinum* blood throughout the period of larval growth in which determinations were made. The figure for non-protein nitrogen, 5.80 mg./cc., corresponds

to that which would characterize the blood of *A tigrinum* larvae of roughly 64 mm. body length, as judged by interpolation on the Table for the latter species. It is interesting to note that 38 mm. and 64 mm. represent essentially equivalent growth stages for *A. punctatum* and *tigrinum* respectively. That is, when larvae of both species are fed maximally from the beginning they will reach these respective lengths at approximately the same time (see curves for growth of the two species, Twitty and Schwind, 1931, Fig. 1). One is led to hazard the prediction that if a complete series of determinations were made on the blood of *A. punctatum* larvae, values very close to those for comparable growth stages of *A. tigrinum* would be revealed. In such event, one would have strong evidence in support of the inference, originally drawn from the results of heteroplastic transplantation, that the blood nutrients are maintained at closely similar levels in the two species of *Amblystoma*.

DISCUSSION

In relating our results to the problem of size-regulation, we may begin by quoting bodily from a recent review article (Twitty, in press) in which they were given preliminary citation.

"The significance of these data for our present purposes depends upon which of the various blood components is most crucial for the synthetic processes of growth. If proteins as such were the principal building material utilized, the evidence obtained would suggest a decline with age, instead of the postulated improvement, in the nutritive opportunities afforded by the blood stream. If on the other hand, as would seem more probable, amino acids are the principal requisite, especially during larval growth when synthesis of new protoplasm is proceeding at such an active pace, the evidence tends to offer strong substantiation for our hypothesis.

"We may thus present as briefly as possible the following conception of the manner in which the growth of an organ is geared with that of the rest of the animal. The eye will be used as an example, although the same rules probably hold for other organs and tissues except as they may be influenced by superimposed factors of function, mechanical limitations of space, etc. As the eye increases in size and physiological age, its cells suffer a gradual loss in assimilative capacity. This is accompanied by a gradual improvement in the nutritive opportunities afforded them by the blood stream. These two sets of changes together provide an effective means of insuring constant size adjustment. The size of the eye at any given moment is a function of its own assimilative capacity and the nutritive opportunities afforded by its environment at that particular stage of growth. Thus in the course of normal development, a state of equilibrium is always in effect. If now the eye

is placed on an older larva, this equilibrium is disturbed, since the graft is exposed to a richer medium for growth than that to which it was previously adjusted. Growth acceleration ensues, and appropriate size relationships are restored. It is important to bear in mind, however, that a nutritive differential alone would not account for this result. The richer environment enables the graft to exceed its normal growth rate, which is of course an essential feature of the picture. But it is equally important, once the size handicap has been thereby overcome, that this acceleration should be halted. Otherwise the graft would not only overtake the normal organ of the host, but actually surpass it in size. In other words, size-regulation is achieved by a temporary advantage of the transplant, but would be defeated if this advantage were permanent.

"This brings out clearly the indispensable role of the other component involved in growth regulation, namely the decreasing assimilative capacity of the cells themselves. Growth acceleration enables the transplant to overtake the host, but this very acceleration likewise speeds the decrease in its assimilative capacity to the point where its growth advantage disappears, that is, to the same level as the rest of the animal. When this condition is reached, equilibrium is restored. The same mechanisms continue to operate, of course, and it is to them that the size relationships which we observe in normal development are probably to be ascribed."

We believe that some such dual mechanism as that outlined above offers the only satisfactory basis for interpreting the phenomenon of size-regulation. It is perhaps another question whether or not we have properly identified the two components or differentials involved. It is difficult to avoid the conclusion, from the results of transplantation alone, that during larval growth there is in any event a 'falling off' of some kind in the growth potentialities of the organs on the one hand, and an improvement in the opportunities for growth provided by the organic environment on the other. And in support of our identification of the nature of these changes, it may be pointed out that no factors are more likely to play a fundamental role in size limitation than the level of availability of growth materials and the ability of the cells to utilize them. There are of course alternative possibilities. One of these is that the improvement in the growth environment represents, instead of a rise in the concentration of critical nutrients themselves, an increasing concentration or activity of hormones which condition their utilization by the cells. One is well aware of the important role which hormones often play in influencing bodily proportions. There is reason to suggest, however, that this is a secondary or superimposed action, not a

primary part of the essential size regulating mechanism. We know, for example, that size regulation does not "break down" when the pituitary gland is extirpated. The absolute growth rate may be affected, and the proportions of the body somewhat modified (Blount, 1935), but the hypophysectomized larvae attain these new proportions in an orderly and uniform manner. And there is little question that eyes grafted from smaller to larger hypophysectomized larvae would regulate to the size of the hosts' eyes, as in the experiments we have already performed with normal larvae. The relative size of the host's eye, and hence the size to which the graft regulates, might be modified by absence of the hormone, but the new 'norm' for the bodily configuration, whatever it might be, would undoubtedly be restored as effectively as ever. Stated in terms of the basic mechanism as outlined by us, hormones probably modify bodily proportions by a selective influence on the assimilative capacities of the organs and tissues affected. But the general decline in assimilative capacity with age, as well as the rise in nutrient level of the blood stream, probably develop independently of their action. It is probable that other factors which influence relative size, such as "functional burden" in the case of the heart, also produce their effects by *acting upon*, not by *replacing*, the same basic differential.

Although there is a considerable literature on chemical changes in the blood associated with age (McCay, 1939), there appears to be little with which the present data can be directly compared. The percentage of total nitrogen (Swanson and Smith, 1932) and total proteins (Toyama, 1919) increases with age in the rat, and Wells (1913) found an increase in proteins in the blood of rabbits. According to Toyama, the percentage of non-protein bodies remains quite constant in the rat. Significant comparisons probably cannot be made, however, until data are available for different animal types on the changes throughout life. In view of the differences in the mode of development or life history between various vertebrates, as for example between amphibians and mammals, it is difficult to judge which stages in each (larval, fetal, etc.) might be regarded as comparable. We shall perhaps gain some information on these points by extending our study, as is planned for the near future, into and beyond the stage of metamorphosis.

The hypothesis outlined here, although obviously still tentative, may permit a new orientation to the problem of proportionate growth, and possibly afford a framework for more comprehensive study. The general validity and applicability of the concept presented will depend in part on the extent to which pertinent data already available from a wide variety of fields can be successfully harmonized with it.

SUMMARY

The concentration of sugar, total nitrogen and non-protein nitrogen was determined on blood samples taken from larvae of *Amblystoma tigrinum* in successive stages of growth. The concentration of sugar and total nitrogen remains essentially constant throughout the period of larval growth encompassed by the study, while non-protein nitrogen registers a marked increase with age. Direct measurement of amino acid nitrogen on two of the samples shows that the rise in non-protein nitrogen represents primarily an increase in amino acids. Fragmentary data on the blood of the slower growing *A. punctatum* indicate that at equivalent stages of larval growth, the concentration of blood nutrients is very similar in both species of *Amblystoma*. On the basis of these data and the results of previous transplantation experiments an hypothesis is constructed concerning the nature of factors responsible for the regulation of proportionate growth.

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THE GROWTH-ACTIVATING EFFECT OF EXTRACTS OF ADULT AND EMBRYONIC TISSUES OF RAT ON FIBROBLAST COLONIES IN VITRO*

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A paper recently issued from this laboratory (Hoffman, Tenenbaum and Doljanski, 1940) described the stimulating effect of aqueous extracts from tissues of adult chickens on growth of cell colonies *in vitro*. In continuation of these investigations the growth-stimulating effect of aqueous extracts from tissues of adult rats was tested quantitatively.

PROCEDURE

Preparations of tissue extracts:

Organs were removed from animals under sterile conditions. Extracts were prepared by mincing the tissues finely with scissors and then adding Tyrode solution in proportion 5:1, shaking well, and allowing to stand in the refrigerator for 24 hours. After this time the mixture was centrifuged and the supernatant fluid pipetted off.

Tissue culture technique:

The effect of organ extracts on the growth of tissue cultures was tested on chicken fibroblast colonies maintained in Carrel flasks. Experiments were carried out as follows: A 3rd passage hanging-drop culture was divided into two equal parts. One half was planted in a Carrel flask containing chicken plasma diluted with Tyrode solution in proportion 1:2 and used as a control—the other half was planted in the same medium using the tissue extract to be tested as the fluid phase. The fluid phase was not changed during the course of the experiment. Each experiment extended over a period of from 6 to 8 days. Growth rate curves of control and experimental cultures were plotted from planimetric measurements of surface area according to the method of Ebeling.

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EXPERIMENTAL

The effect of aqueous extracts of the following organs from adult rat on the growth of chicken fibroblast colonies *in vitro* was tested: brain, heart, kidney, liver, lung and spleen. The number of experiments carried out with extracts of each organ varied between 8 and 27. Figures 1, 2, 3, 4, 5 and 6 present in typical curves results obtained with each organ extract. Tables I, II, III, IV, V and VI contain the numerical basis for these curves.

Extracts of rat spleen, liver, kidney and lung decidedly inhibit growth of fibroblast colonies. Heart extract has neither a growth-stimulating nor inhibiting effect. Brain extract possesses very pronounced growth-stimulating properties.

As part of these investigations, a study was undertaken of the action of aqueous extracts of rat embryos on the growth of chicken fibroblasts *in vitro*. Extracts of 7 to 12 day old rat embryos were prepared in the same way as extracts of adult organs, and used in the same concentration. The results are given in Fig. 7 and Table VII.

Rat embryonic extract has a marked growth-stimulating effect on the growth of chicken fibroblast colonies in vitro.

RESULTS AND DISCUSSION

The results of the experiments here detailed can be summarized as follows:

(a) Of all the extracts of organs and tissues of adult rats tested, only brain has a growth-stimulating effect on chicken fibroblasts *in vitro*.

(b) Extracts of all other organs of adult rats tested have no growth-stimulating properties whatsoever.

(c) Extracts of kidney, liver, lung and spleen definitely inhibit the growth of cell colonies.

(d) Extracts of rat embryos markedly stimulate the growth of chicken fibroblasts *in vitro*.

In comparing the growth-promoting capacities of extracts of organs of adult rat with those of adult chicken, it is evident that the extracts of most tissues of these two species influence the growth of cell cultures quite differently.

As was previously shown (Hoffman, Tenenbaum and Doljanski, 1940), all organs of adult chicken contain factors which stimulate cell growth. Although they varied as to degree of activity, the extracts of all the organs tested (brain, bone-marrow, liver, lung, smooth muscle, pancreas, kidney, ovary, oviduct, spleen and testis) stimulated the

growth of fibroblast colonies. The extracts of most rat organs are devoid of growth-stimulating properties. The differences between growth-promoting properties of the tissue extracts of chicken and rat are very evident in the case of heart. Heart extract of chicken has very marked growth-promoting properties whereas that of rat has no effect whatsoever on growth of cell colonies. These differences are still more striking with extracts of some other organs tested—extracts of chicken liver, lung, spleen and kidney stimulate growth whereas extracts of these rat organs definitely inhibit it.

The growth inhibition described here as due to rat extracts is much more definite than the inhibition discussed in connection with the action of extracts of certain chicken tissues. In a previous paper (Hoffman, Tenenbaum and Doljanski, 1940) it was shown that, in contrast to some organ extracts which have very pronounced stimulating properties, other chicken organ extracts have relatively slight ones. It was pointed out that these differences may be due to the presence of certain factors in these organs which counteract growth stimulation. However, in chicken even in the case of liver and kidney extracts, in which cultures grow relatively poorly as compared to brain and heart extracts, the stimulating effect is always pronounced. In the rat, on the contrary, inhibition is not relative but absolute. Cultures growing in media containing extract of rat organs which inhibit cell growth are always smaller than those in control medium (Fig. 8a and b).

The investigations reported in this paper demonstrate furthermore that in rat, apart from extracts of embryos, only brain extracts are able to stimulate cell growth. The brain occupies a unique position among the tissues of adult rat. Its stimulating properties are almost as great as those of chicken brain extract and it can therefore be said to be one of the most potent cell growth-stimulating extracts we know. The mean size of cultures grown in medium containing rat brain extract is 62 sq. mm. on the 6th day of growth. Some cultures attained to 114 sq. mm. in this period (Fig. 9a and b).

In view of our limited knowledge of the factors involved in cell growth it seems premature to attempt an explanation of the singular position of brain among adult rat organs. The fact that the extract of an organ such as brain which is most stable with regard to mitotic activity should have such pronounced growth-stimulating powers speaks clearly against any notion that it is the state of proliferation of the tissue which determines its growth-stimulating capacity.

Mention must be made of the fact that in these experiments the effect on growth of adult and embryonic rat tissue extracts was tested on chicken fibroblasts—that is to say on a heterologous material. This procedure was necessary because until now we have not succeeded in

obtaining a strain of rat fibroblasts suitable for quantitative investigations despite numerous attempts. However, the results of these experiments show clearly that heterology does not interfere with growth-promoting activity. The striking growth-stimulating effect obtained with extracts of rat brain on the growth of chicken fibroblasts demonstrates clearly that a heterologous material is effective if it contains growth-activating factors.

Further experiments were carried out in order to test the effect of heterologous extracts on cell growth. The growth-promoting action of embryonic extract of rat was compared with that of embryonic extract of chicken on chicken fibroblasts. These experiments showed that the rat extract is not at all weaker, but in many cases stronger than the chicken extract (Fig. 10 and 11a and b). These results correspond to those of Carrell and Ebeling (1933). These authors investigating the action of heterologous embryonic extracts on fibroblast cultures found that the embryonic extract of mouse, rabbit and guinea-pig possess the same growth-stimulating properties as chicken embryonic extract.

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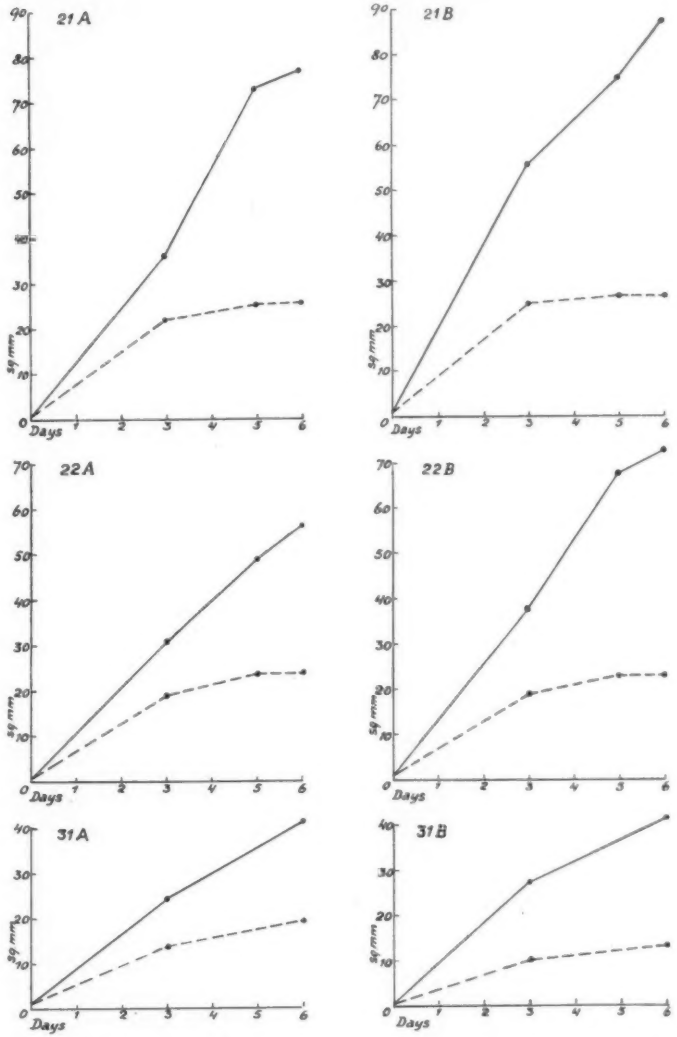

Fig. 1. Growth rate of sister halves of chicken fibroblast cultures growing in brain extract and in protective media.

——— brain extract medium

– – – – protective medium

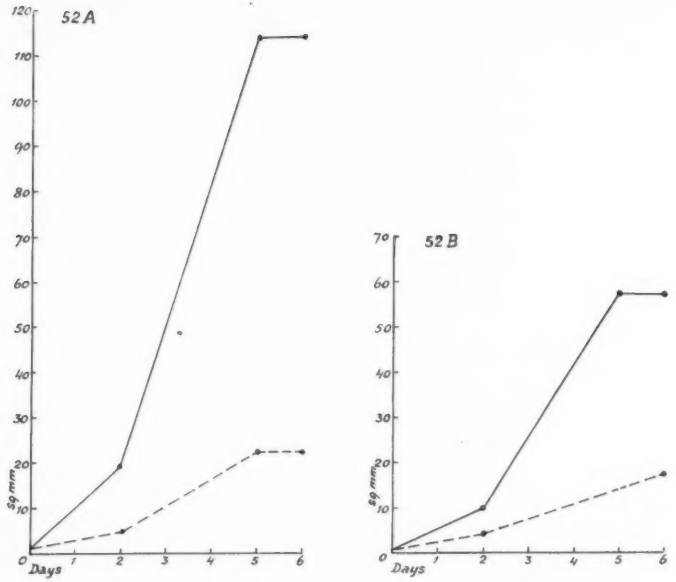

Fig. 1, continuation. Growth rate of sister halves of chicken fibroblast cultures growing in brain extract and in protective media.

——— brain extract medium

– – – – protective medium

TABLE I
(TO FIGURE 1)

No. of Culture	Protective Medium Size in sq. mm. on 2nd	4th	6th day	Rat Brain Extract Medium Size in sq. mm. on 2nd	4th	6th day
21A	15	24	26	25	53	77
21B	17	26	27	37	65	88
22A	12	21	23	20	39	56
22B	13	20	23	25	52	72
31A	10	15	19	15	30	41
31B	7	11	13	18	32	41
52A	5	17	22	19	80	114
52B	4	11	18	10	40	57

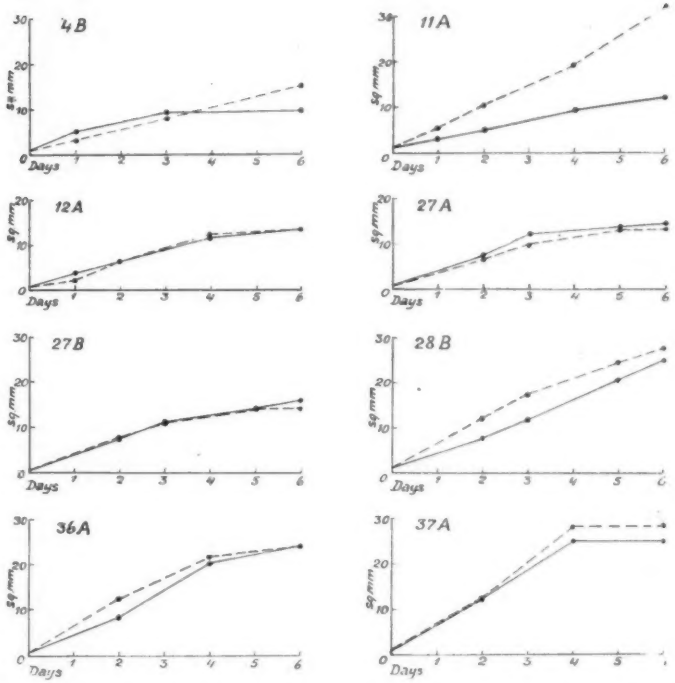

Fig. 2. Growth rate of sister halves of chicken fibroblast cultures growing in heart extract and in protective media.

———— heart extract medium

– – – – protective medium

TABLE II
(TO FIGURE 2)

No. of Culture	Protective Medium Size in sq. mm. on 2nd	4th	6th day	Rat Heart Extract Medium Size in sq. mm. on 2nd	4th	6th day
4B	5	11	15	7	9	10
11A	10	19	32	5	9	12
12A	6	12	14	6	12	14
27A	6	11	13	7	13	14
27B	7	12	14	7	12	16
28B	12	21	28	8	16	25
36A	13	22	25	8	21	25
37A	12	28	28	12	25	25

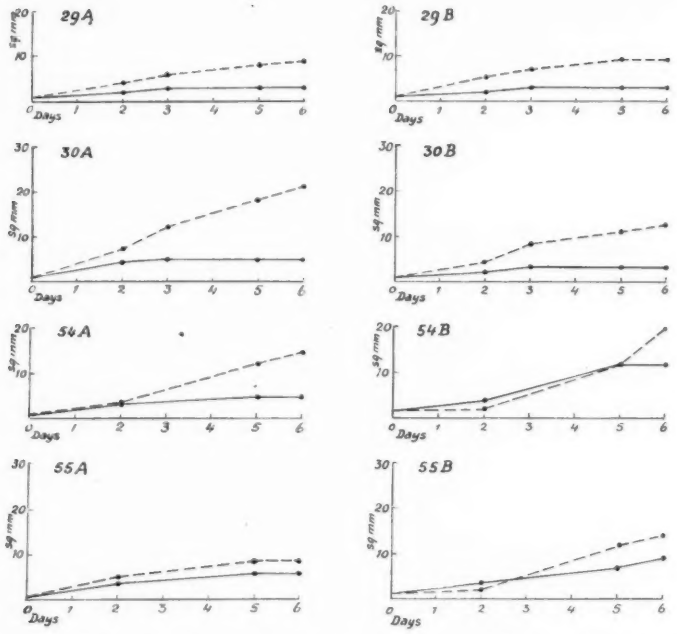

Fig. 3. Growth rate of sister halves of chicken fibroblast cultures growing in kidney extract and in protective media.

———— kidney extract medium

– – – – protective medium

TABLE III
(TO FIGURE 3)

No. of Culture	Protective Medium Size in sq. mm. on 2nd	4th	6th day	Rat Kidney Extract Medium Size in sq. mm. on 2nd	4th	6th day
29A	4	7	9	2	3	3
29B	5	8	9	2	3	3
30A	7	15	21	4	5	5
30B	4	10	12	2	3	3
54A	4	9	15	4	5	5
54B	2	9	20	3	9	12

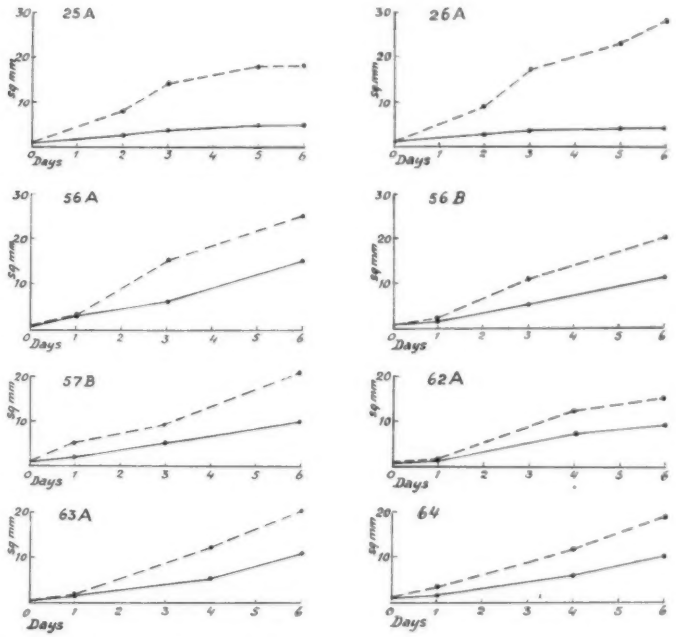

Fig. 4. Growth rate of sister halves of chicken fibroblast cultures growing in liver extract and in protective media.

——— liver extract medium

– – – – protective medium

TABLE IV
(TO FIGURE 4)

No. of Culture	Protective Medium Size in sq. mm. on 2nd	4th	6th day	Rat Liver Extract Medium Size in sq. mm. on 2nd	4th	6th day
25A	8	11	13	3	4	6
26A	9	20	28	3	4	5
56A	9	18	25	4	9	15
56B	8	14	20	3	7	11
57B	7	13	21	3	7	10
62A	5	12	20	3	7	9
63A	5	12	20	2	5	10
64	6	12	19	3	6	10

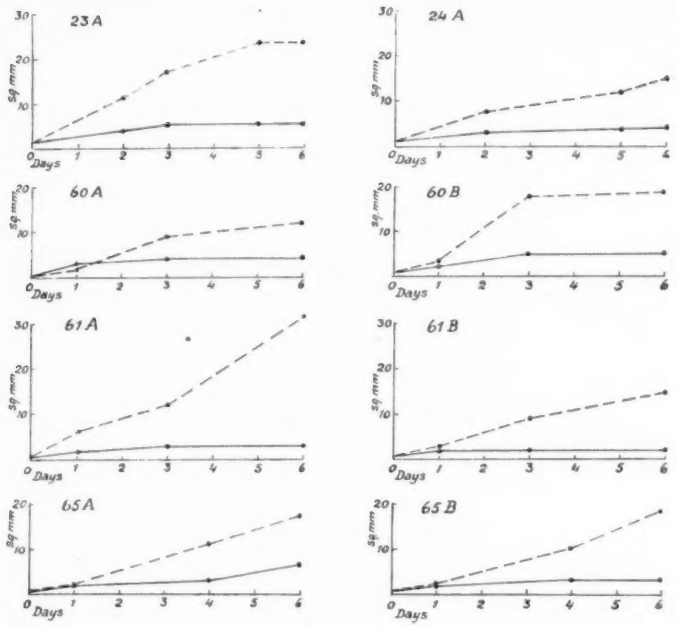

Fig. 5. Growth rate of sister halves of chicken fibroblast cultures growing in lung extract and in protective media.

———— lung extract medium

– – – – protective medium

TABLE V
(TO FIGURE 5)

No. of Culture	Protective Medium Size in sq. mm. on 2nd	4th	6th day	Rat Lung Extract Medium Size in sq. mm. on 2nd	4th	6th day
23A	11	20	23	4	5	5
24A	8	11	15	3	4	4
60A	6	11	13	5	5	5
60B	11	18	19	4	5	5
61A	9	19	32	2	3	4
61B	6	11	15	2	2	2
65A	5	11	17	2	3	6
65B	5	10	18	2	3	3

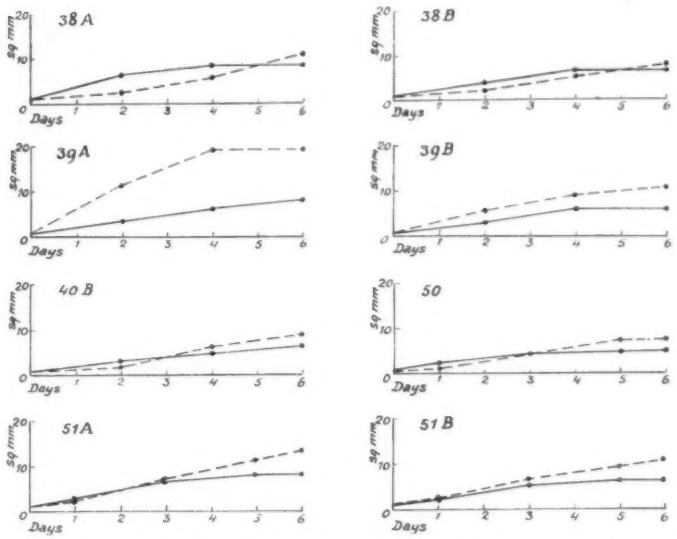

Fig. 6. Growth rate of sister halves of chicken fibroblast cultures growing in spleen extract and in protective media.

———— spleen extract medium

– – – – protective medium

TABLE VI
(TO FIGURE 6)

No. of Culture	Protective Medium Size in sq. mm. on 2nd	4th	6th day	Rat Spleen Extract Medium Size in sq. mm. on 2nd	4th	6th day
38A	2	5	10	6	8	8
38B	2	5	8	4	6	6
39A	11	19	19	4	6	8
39B	5	9	11	3	6	6
40B	2	6	9	3	5	6
50	3	6	8	3	5	5
51A	4	9	13	4	7	7
51B	4	6	9	3	5	6

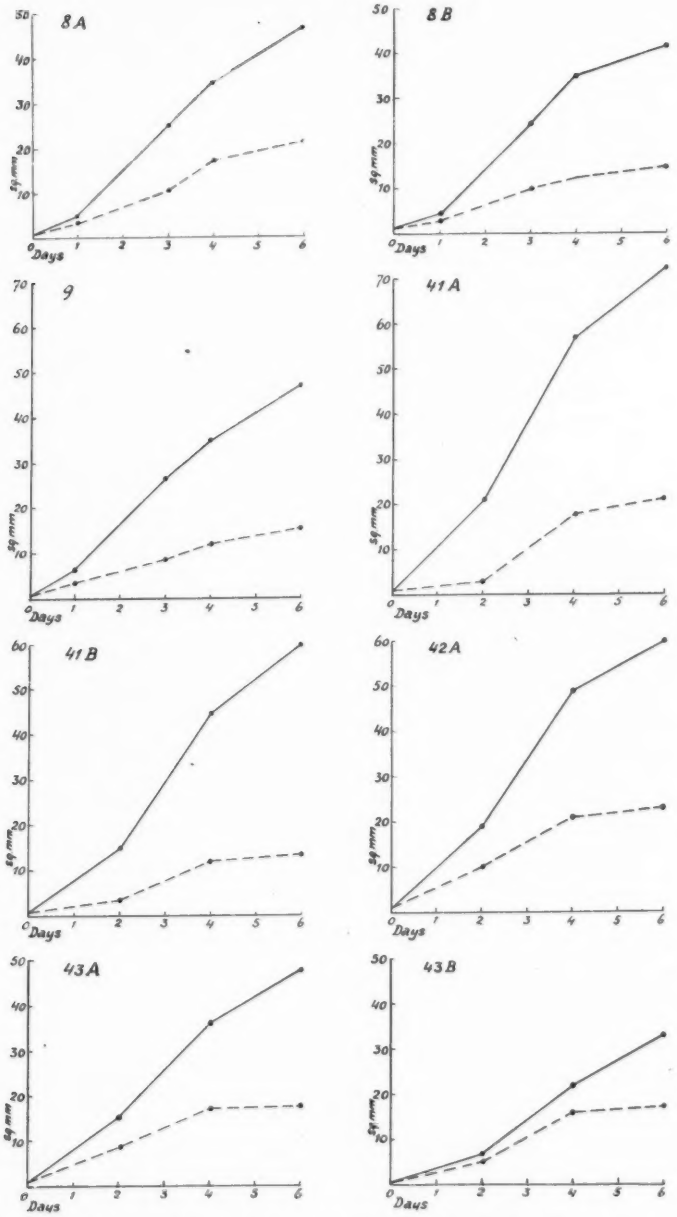

Fig. 7. Growth rate of sister halves of chicken fibroblast cultures growing in rat embryonic extracts and in protective media.

——— rat embryonic extract medium — — — — protective medium

TABLE VII
(TO FIGURE 7)

No. of Culture	Protective Medium Size in sq. mm. on 2nd	4th	6th day	Rat Embryo Extract Medium Size in sq. mm. on 2nd	4th	6th day
8A	7	17	21	14	34	46
8B	6	13	16	15	35	42
9	6	12	15	15	35	47
41A	3	18	21	21	57	72
41B	3	12	13	15	45	60
42A	10	21	23	19	49	60
43A	9	17	18	15	36	48
43B	5	16	17	7	22	33

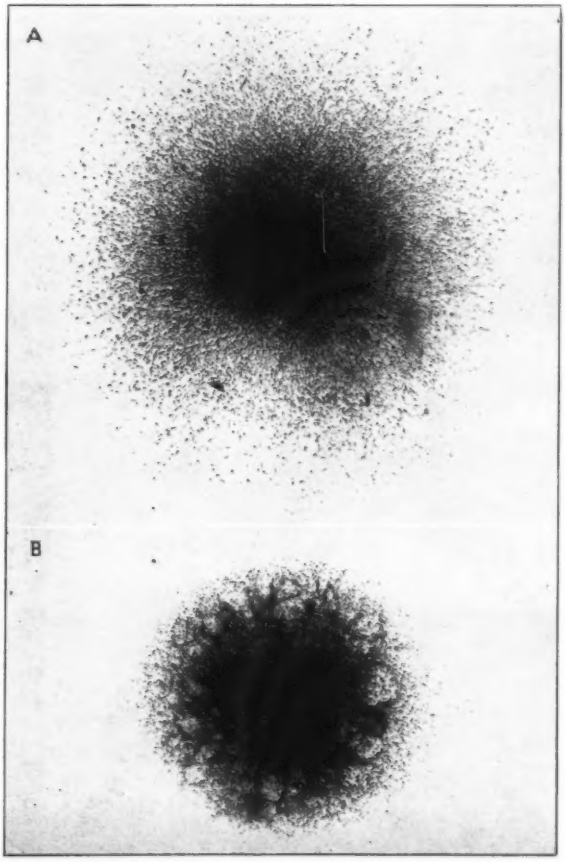

Fig. 8. Experiment No. 51. Sister halves of a fibroblast culture growing in protective (A) and in rat spleen extract (B) media. 7th day. × 12.5, living.

TABLE VIII
(TO FIGURE 10)

No. of Culture	Chicken Embryo Extract Medium Size in sq. mm. on 2nd	4th	6th day	Rat Embryo Extract Medium Size in sq. mm. on 2nd	4th	6th day
15A	22	50	64	15	33	47
15B	21	50	66	11	27	44
44A	6	18	30	15	32	50
44B	15	23	31	11	32	55
45A	15	31	31	14	34	64
45B	16	34	47	13	44	65
46A	12	28	30	18	49	68
46B	15	39	48	18	50	65

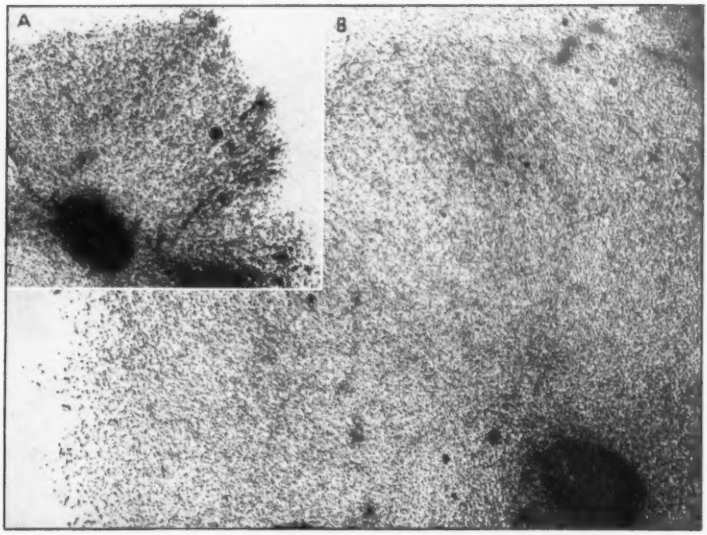

Fig. 9. Experiment No. 52. Sister halves of a fibroblast culture growing in protective (A) and in rat brain extract (B) media.
6th day. × 12.5, living.

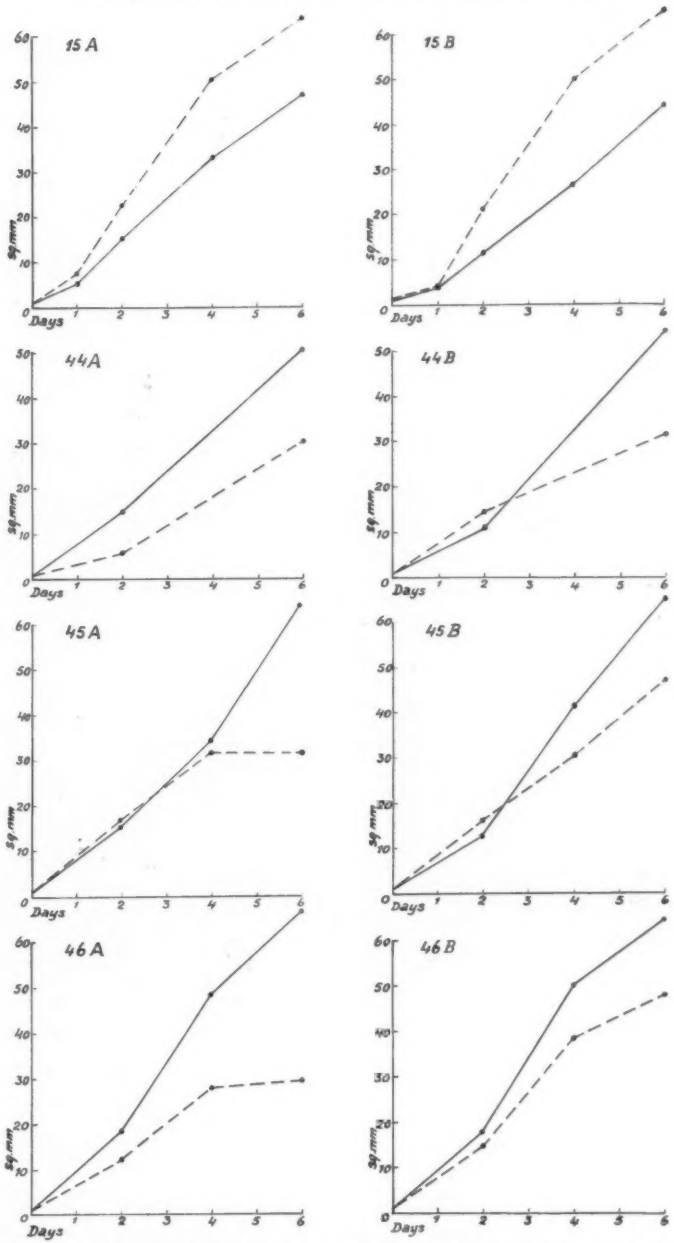

Fig. 10. Growth rate of sister halves of chicken fibroblast cultures growing in chicken embryonic extract medium and in rat embryonic extract medium.
———— rat embryonic extract medium — — — — chicken embryonic extract medium

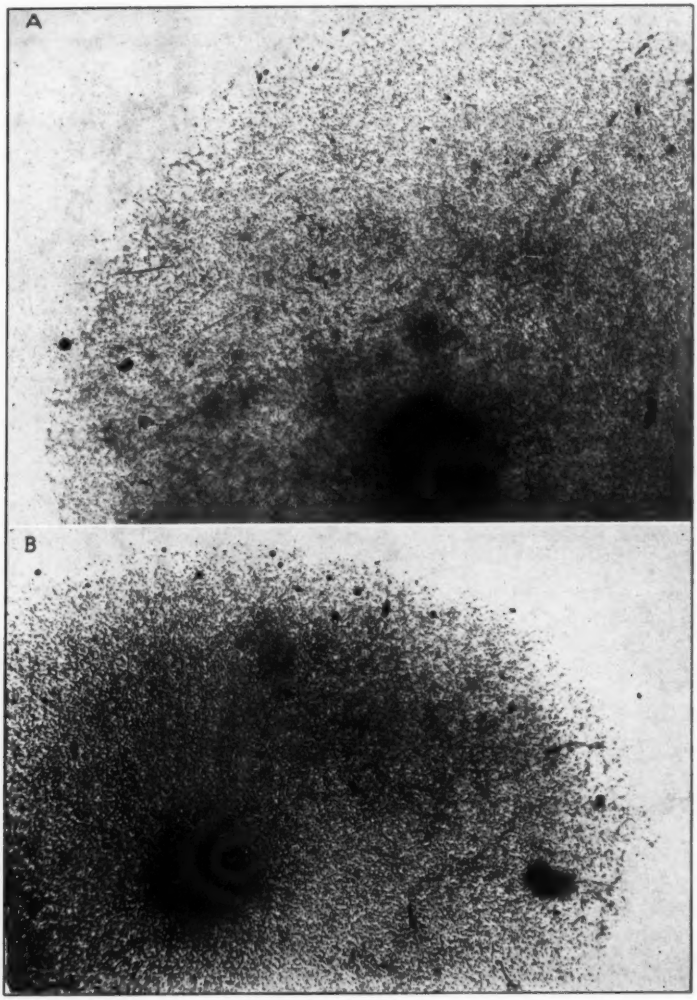

Fig. 11. Experiment No. 46. Sister halves of a fibroblast culture growing in rat embryonic extract (A) and chicken embryonic extract (B) media.
8th day. × 11.5, living.

GROWTH IN VITRO OF THE TESTIS OF DROSOPHILA

By

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(*Received for publication November 1, 1940*)

In the fruitfly, *Drosophila melanogaster,* the shape of the testis undergoes a striking change during development. It is a slightly ellipsoidal vesicle during embryonic, larval, and early pupal stages. During the later part of pupal life it grows into a spiral tube of about two and one half gyres. Most other species of the genus *Drosophila* possess the same type of testis growth though the degree of spiralisation reached in the adult may be as low as only one turn or as high as ten or more turns. *Drosophila virilis virilis* Sturtevant, which together with *D. melanogaster* has been used in these studies, possesses about six gyri.

The form of the testis is determined by that of its thin elastic surrounding membrane and maintained by the pressure exerted on this membrane by the testicular contents. Growth of the testis is primarily due to increase in surface area of the membrane in the region of its posterior end. It proceeds slowly and symmetrically up to about one third of the pupal period (Kerkis, 1931) where it increases in rate rather abruptly and also becomes asymmetrical, leading to spiralisation (Fig. 1). A causal factor in the change in growth and form of the testis is the vas efferens. This duct during early pupal life develops from an imaginal disc which originally lies separated from the gonad. After growing toward the posterior end of the testis, duct and gonad join in the region of the terminal growth zone of the testis. It is at this stage that spiral growth sets in. Results of various types of transplantation experiments have led to the interpretation that the growth of a testis after attachment to the vas is governed by a hypothetical growth substance which is given off by the vas in different amounts to opposite sides of the testis (for a preliminary account see Stern, 1940; full paper in preparation).

A further analysis of this situation seemed possible if observations of the phenomena of testis growth could be made *in vitro.* Although insects are reputed not to be favorable objects for tissue cultures, an attempt was made to explant whole testes during various developmental

*Aided by a grant from the Women's Auxiliary of the Lankenau Hospital Research Institute, Philadelphia.

stages and to see whether growth would proceed. Hope for success was not only based on the fact that some workers have reported positive results before (Goldschmidt, 1915-1917; Frew, 1929; Fischer and Gottschewski, 1939; Gottschewski and Fischer, 1939), but also because the testis appears to grow by elongation of its sheath without nuclear division. The following is a preliminary report on the method employed and of the positive outcome of some basic experiments.

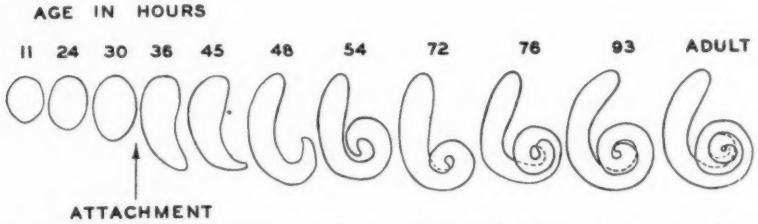

Fig. 1. *Drosophila melanogaster*. Growth of testis during pupal stages. The times given represent hours after beginning of pupation (at 25° C.). Attachment of vas efferens to testis occurs between 30 and 36 hours of pupal life. (c. 22 ×).

METHODS

The culture medium consisted of filtered, boiled seawater diluted with four parts of distilled water and used either alone or with the addition of the histolyzed contents of pupae. This medium may be regarded as a well buffered solution. Since the addition of pupal tissues appeared to make little difference in the behavior of the testes, its use was abandoned in later experiments, as was also the use of more strongly diluted sea water (one in five parts of distilled water) which had been tried in a few experiments without producing noticeably better results than the more concentrated solution.

Pupae of varing ages were washed in 96 per cent alcohol, placed in a drop of salt solution on a depression slide and dissected with steel needles. For some time prior to attachment to the vasa efferentia, the testes are completely free from surrounding tissues. They were either transferred by means of a pipette to another depression slide or left on the original slide in a mixture of salt solution and the hystolyzed contents of from one to three pupae. The cultures were placed individually into moist chambers (Petri dishes). Temperature was not controlled.

It is obvious from this description that no attempt was made to maintain bacteriological sterility although all glassware and solutions were boiled or sterilized before use. It should not be difficult to exclude infections completely. However the methods proved to be adequate to permit survival and development of the testes for forty-eight hours or longer without, in most cases, appreciable bacterial growth. Further-

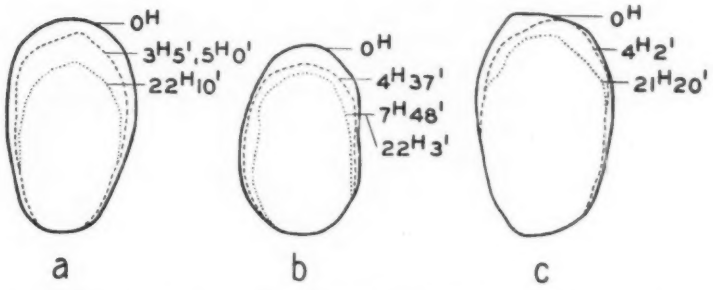

Fig. 2 Explants from early pupae before attachment to vas efferens has occurred. Outlines drawn at various intervals. O^H signifies initial stage, other times give hours (H) and minutes (′) after beginning of experiment. (c. 76 ×).

a, b. *D. melanogaster*
c. *D. virilis*

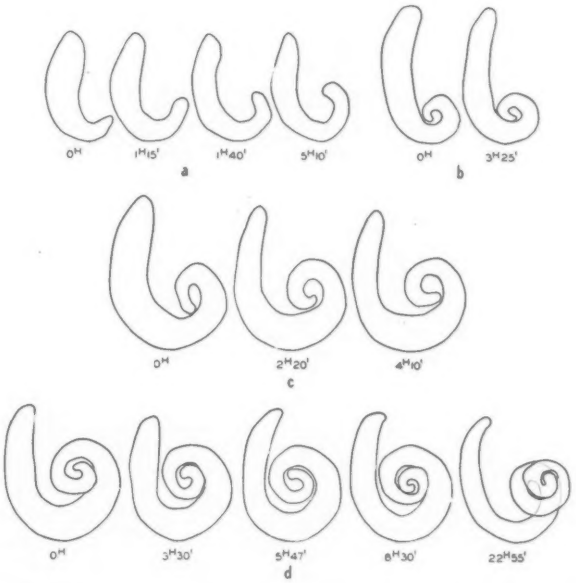

Fig. 3. Explants from pupae of varying age, after attachment to vas efferens had occurred. (c. 25 ×).

a, b. *D. melanogaster*
c, d. *D. virilis*

more, essential changes of testis shape could be observed within two hours, so that during this preliminary work no need arose for a more refined technique. Outline drawings of all explants were made by means of a camera lucida at from one to twelve hour intervals. More than 150 explanted testes of *D. melanogaster* and about 100 of *D. virilis* were studied.

In a general way testes were judged to be alive as long as they remained transparent which was usually for two or more days after explantation.

RESULTS

Explantation of ellipsoidal gonads removed before and up to the time at which attachment to a vas efferens would have occured never led to growth. Generally, no visible change of any kind occurred in these testes during the first two hours *in vitro,* after which they underwent continuous decrease in volume without any striking changes in form (Fig. 2).

Explantation of testes in various stages of spiralisation, beginning with those corresponding to the 45 hour type indicated in Fig. 1, permitted typical spiral growth to continue. Within one hour after explantation, elongation and curving of the end of the testes was observed. As *in situ,* the rate of growth was higher in *melanogaster* than in *virilis* explants. This growth led to the addition of sometimes one whole gyre and to widening of the funnel-shaped growing end-section as in normal growth (Fig. 3). A decrease in diameter of the older parts of the testes accompanied these changes. This decrease may have been due partly to processes of shrinkage similar to those observed in young ellipsoidal explanted gonads and is probably not caused by the osmotic properties of the salt solution, which if not isosmotic with the testes was hypo- rather than hyper-tonic. It is likely that at least part of the decrease in diameter of the older parts of a testis is a result of the terminal growth of its membrane, the new space thus created becoming occupied by germ cells which before have filled a smaller volume and exerted pressure on the membrane. Though exaggerated *in vitro,* this narrowing of the older parts of the testis during early spiral growth is typical for changes of form *in situ* (Fig. 1; compare the stages from 30 hours to 54 hours with each other). In these processes of membrane growth no simultaneous influence of the vas efferens was observed. Whether a testis had been explanted together with this duct or with the whole duct system or whether it had been completely isolated seemed to have no bearing on its further development. Occasionally in cases where no duct was present, growth was slightly irregular so that it did not result in a typical spiral; but it seems pre-

mature to draw conclusions as to the rôle of absence of duct in these rare cases.

During the first hours, after the attachment of the testis to the vas efferens begins, there occurs a critical period in the interrelation of these organs. Here appreciable growth *in vitro* seemed to proceed only when they remained together. When of a pair of testes from the same individual, one was left joined to the duct and the other was separated

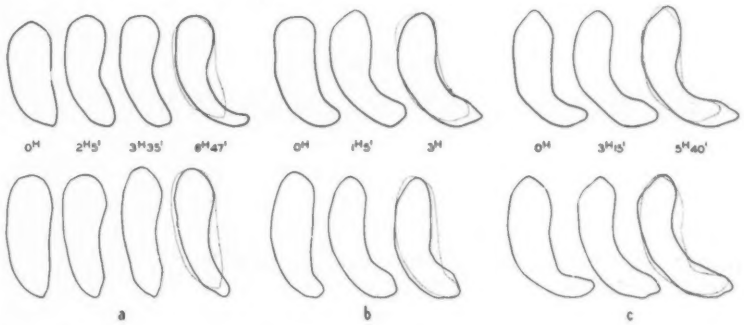

Fig. 4. Explants from pupae during the period of attachment to vas efferens.
upper row: testes remained in contact with vas.
lower row: testes from same individuals, during explantation separated from vas.

The dotted lines represent copies of the intial stages (O^H) to facilitate comparison. (c. 25 ×).

a. *D. melanogaster*
b. c. *D. virilis*

from it, typical growth occurred in the former gonad while none or slight growth only seemed to proceed in the latter (Fig. 4). This difference in the behavior of the testes is hardly due to injury during separation from the duct, because junction of the organs during this period is so superficial that they easily fall apart without any visible damage. It is, however, difficult to obtain many cases of this type of experiment, as the majority of explants of this particular age group undergoes early disruption at one or more places of their growth zone leading to extrusion of germ cells and termination of organ development.

For a closer analysis of these phenomena it will be desirable to produce *in vitro* junction of gonads and ducts at different stages at will. It is possible to place originally separate organs so that they touch each other in culture. If held together, by means of small fragments of coverglasses, their tissues join within less than one hour

in a manner similar to typical development. Sufficient data are not yet available regarding the interaction of such artifically combined systems.

SUMMARY

A preliminary report is made of a simple method of cultivating *in vitro* testes and genital ducts of two species of *Drosophila*. If explanted at suitable stages, typical growth and form changes proceed. Present data suggest a critical stage during which the vas efferens promotes growth of the gonad.

The work was done at the Marine Experimental Station of the Lankenau Hospital Research Institute.

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IN VITRO CONDITIONS FAVORING ECDYSIS AT THE END OF THE FIRST PARASITIC STAGE OF HAEMONCHUS CONTORTUS (NEMATODA)

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(*Received for publication November 12, 1940*)

The pathogenic nematode, *Haemonchus contortus,* which lives as a parasite in the fourth or true stomach (abomasum) of its host, is found in ruminants throughout the world. In common with related forms, such as hookworms, this "twisted wireworm" has four larval stages, the termination of each of which is marked, morphologically, by ecdysis, much as in insect growth.

The first two of the larval stages are obligate free-living forms, feeding on bacteria in the host feces in which the eggs have been conveyed outside. At the end of this early two-stage development the larvae are, for the first time, infective, and are unable to feed or advance further until they reach the host in which they are to be parasitic. In the case of *H. contortus* the infective larvae migrate from the sheep feces to blades of grass and other forage plants, and are thus conveniently placed for ingestion by the grazing animal. Until passively transferred back to their parasitic environment, they are to a certain extent protected by the presence of a cuticular sheath, which forms a loose coat around the infective 3rd stage larva within. It is possible that this sheath may be lost early after ingestion, perhaps in the ruminant's mouth. All later development in this species is believed to take place on the wall of the abomasum. The parasitic phases include development of the 3rd stage larva to its ecdysis freeing the 4th stage, growth (3 to 6 fold increase in length) and development of the 4th stage to its ecdysis freeing the young adult, with final growth (another 3 to 6 fold increase in length) and development of the adult to the reproductively mature worm.

Attempts have been made by Glaser and Stoll (1938a) to bring this entire life cycle to pass in test tubes under conditions of bacterial sterility. "Culturing" of the normally free-living stages of strongyloid worms of this sort naturally in feces is not difficult. As a step toward culture of the parasitic stages Glaser and Stoll (1938b) first reared these free-living stages without bacterial contamination in agar-yeast-liver extract media. Efficient infective larvae were developed, and confirmed as such by their ability to parasitize sheep. As far as further bacteria-free culture was concerned, such sterilely grown infective

larvae appeared to offer no advantage, however, over growing them to the infective stage in the usual nonsterile fashion and then chemically sterilizing them (Glaser and Stoll, 1940). Utilizing this preliminary procedure, it was possible to initiate parasitic growth and carry it forward in culture to the end of the 2nd parasitic (4th larval) stage (Glaser and Stoll, 1938c). In such cultures no adults were found, and advanced forms were usually encountered dead or moribund. Of considerable significance nevertheless was the fact that the block at the end of the free life had been broken under in vitro conditions and true parasitic development induced.

With the problem offering a certain refractory character at this level, the author undertook to explore more particularly some of the factors influencing initiation of the parasitic phase itself (i. e. the changes culminating in ecdysis at the end of the 3rd stage) as a step in a better evaluation of the rôle of different culture conditions and ingredients. The present report deals primarily with salt solutions and liver extract, indicating those of choice, with the selection of their optimum concentrations and conditions of test.

MATERIALS AND METHODS

Source of Infective Larvae.

To secure and maintain a supply of *H. contortus* infective larvae, a pure infection with these parasites is established (Stoll, 1929) in a lamb or sheep reared helminth-free after the method of Smith and Ring (1927). Such infections can be maintained serially, each lasting for a variable number of weeks, depending on condition of the host, number of larvae introduced into the sheep, etc. There is no increase in the number of parasites within the host. Infections of suitable size are established by administering by mouth a single dose of 60 to 200 larvae per kg. of host weight, i. e., 3000 to 5000 infective *Haemonchus* larvae to an animal of 25-50 kg.

Various methods have been devised to get good yields of infective larvae from sheep feces, but the simplest one, which is approximately the natural one, turns out to be best. This is to collect feces from the infected sheep directly into paraffined paper boxes, add a small amount of water, cover and set aside on the laboratory table (Sarles, 1932). Free-living nema contaminants are avoided by preventing contact of fecal pellets with the rump wool of the host, and the use of distilled water. At room temperature 7-10 days are required for larvae to develop to the infective stage. With 50-90 grams of fecal pellets thus "cultured" in a half-pint box, yields of infective larvae, isolated after 10-20 days, range from 30-85 per cent of the number of eggs. Larvae may thus be secured in abundance, if the source animal has an infection yielding 2000 to 4000 eggs per gram (Stoll, 1930).

The larvae are freed from the fecal mass by using the Baermann isolation technic. Due to the abundance of mold spores in the fecal "culture" at the time of isolation, Baermann funnels are best placed in a room distant from the one where the later sterile technics are undertaken. If the larvae secured at isolation are found to contain undeveloped or dead specimens, attempts to sterilize them result unsatisfactorily.

Preparation of Larvae for in vitro Experiments.

With infective larvae in good condition, the method of Glaser and Stoll (1940), using Labarraque's solution of sodium hypochlorite, has been routinely successful in freeing them from the sheaths which mark the termination of the 2nd stage and securing the 3rd stage forms bacteriologically sterile. A single simplification has been developed in this technique. As one step, the larvae are repeatedly allowed to settle through columns of sterile tap water in order to wash from them the microorganisms not killed by the hypochlorite, and those additional bacteria gradually released from their intestinal tracts. Instead of using for this, long tubes with constricted ends which are later flame-sealed after filling with water, sterile bacteriological test tubes (18 × 160 mm.) are now employed. These permit the use of 10 ml. of sterile tap water through which the larvae settle to the bottom of the tubes. Following gravity washing as the larvae fall faster than organisms freed from them, the supernate is withdrawn and the larvae are transferred to successive tubes for further washing in the same fashion. About three times as many sterile water washings are required in using the test tubes. The entire process is carried out at room temperature.

Sometimes as much as half the original number of larvae are lost through clumping and lagging in sedimentation during the sterilizing procedure, but 20,000 to 40,000 or more may be brought through the 3-day process successfully in one lot. After sterilizing, and until needed, they are held in the refrigerator at 5° C in a shallow layer of dilute Tyrode solution in 50 ml. Erlenmeyer flasks. All larvae are tested for sterility before use in experiments.

So far no consistent differences have been found in the ability of the larvae to undergo their first parasitic ecdysis in vitro as regards their age in culture before chemically sterilizing them, or their age in the refrigerator afterward,—provided vigorous larvae are used. Larvae for inoculation of culture tubes have not been employed unless 95 to 100 per cent of a fair sample of the sterile lot are alive and wriggling. In one test of the relation of the "physiological age" of sterile larvae to their ability to undergo ecdysis later, no difference could be noted between one half of a lot which had been refrigerated 5 days, and the other half of the same lot which had been incubated in a

flask in shallow, dilute Tyrode solution at 38½° C for 5 days, the lots then tested in parallel in Tyrode-liver extract and Ringer-liver extract media.

The use of exsheathed, infective larvae free from contamination underlies all subsequent experimentation, which in every detail (except final examination of larvae after incubation) is carried out under conditions of bacteriological sterility. For those whose previous experience with parasites has taken account of only helminthological sterility, the exclusion of contaminating bacteria and fungi in cultures will have numerous disappointments. Due to the large number of tubes to be filled with media and inoculated in single experiments, the avoidance of windy days in beginning an experiment has, for instance, proved a saving precaution in these tests.

When tubes have been sealed after inoculation, "Parafilm" has been used over the cotton plugs trimmed down even with the top of the tube.

PRELIMINARY OBSERVATIONS

As mentioned, the infective *Haemonchus* larva at the end of the free-living period and in its 3rd stage is enclosed in a loosely fitting sheath which originally formed the cuticula of the 2nd stage larva. In the sterilizing process with sodium hypochlorite solution the larva is first set free from this sheath, and then the sheath itself dissolves. Under conditions of natural parasitism this larva would have been ingested by the sheep while still within the 2nd stage sheath. The freed 3rd stage larva thus constitutes the bridging form which comes at the end of life in the open and at the beginning of life in the host. But unless external conditions are appropriate, no further change takes place in such a larva, and it either dies after exhaustion of its stored nutriment or succumbs earlier to some hazard of its environment. Lapage (1935), despite his ability to sterilize and exsheath the infective larvae of *Haemonchus* and other strongyloid forms, reported only 10 parasitic ecdyses in more than 1200 chemically exsheathed 3rd stage larvae studied, and "the occurrence of these third ecdyses did not appear to be related to the composition of the media in which they occurred."

Under proper conditions, however, the larvae do proceed to develop to the 4th stage. Then one observes that:

1. As the 4 stage develops it is seen to become gradually free from the 3rd stage sheath, first at head and tail, eventually throughout its length. This is evidently not so much due to enlargement of the sheath as to a gradual decrease in size of the young parasite. In one series of measurements, in which unmodified 3rd stage larvae averaged 570μ in length, ten 4th stage larvae, still ensheathed, measured 415μ, their intact sheaths 580μ, representing a decrease in length of over one-

fourth. The impression is gained that in tubes showing ecdysis most promptly, the pre-ecdysis decrease in size is less.

Lapage (1935) observed that "parasitic larvae are often retracted in their sheaths," but refers to it as "shrinkage and distortion", "an osmotic effect." The decrease seems better interpreted as due primarily to the utilization of stored food material during the period of initial parasitic development, which, as a sort of "pupation" ending in ecdysis, results in a smaller but more advanced larva. As shown later, ecdysis occurs in three-salt Ringer solution containing only inorganic constituents, and to a very small degree even in distilled water. Exogenous food sources are thus evidently not necessary to initiate the parasitism, as Lapage (1935) speculated and the present studies appear to prove. While Veglia (1916), examining recently parasitized sheep, noted "little or no growth in the third stage" of *Haemonchus*, the present observation of initial decrease is made more readily in vitro than in vivo. Miller (1939) found a decrease in size averaging about 1/5th in length (from 1.6 to 1.3 mm.) in the trematode *Postharmostomum laruei* "after it has been in the [mouse] host 30 hours." Ferguson (personal communication) has observed a similar average decrease in length of about 1/5th in another trematode, *Posthodiplostomum minimum*, during its first few days in the bird host. It is possible this is a common phenomenon in the first stage of helminthic gastro-intestinal parasitism.

2. The mouth of the larva undergoes marked modification, the smoothly rounded anterior end becoming noticeably blunt, even when observed under low power magnification. This is due to the development of the provisional buccal capsule. To quote Veglia again (1916): "The object of this period of life seems to be the formation of a mouth apparatus adapted to piercing the mucosa of the stomach." The change is clearly shown in Fig. 1, where comparison may be easily made between the rounded anterior end of the sheath representing 3rd stage morphology, and the blunt mouth of the 4th stage larva within. (The curved line connecting the two is the cuticular lining of the earlier esophagus.)

There is no change in the posterior end of the emerging 4th stage larva except for the tail to be held slightly at an angle to the main axis of the body. This is in contrast to the change from the long attenuated kinked tail characterizing the 2nd stage to the stubbier tip of the 3rd stage, as illustrated in an earlier article (Glaser and Stoll, 1940).

3. When the morphological changes are completed ecdysis occurs through the escape of the young 4th stage breaking through the old sheath. Break-through characteristically occurs not at the old mouth opening, as Lapage (1935) also points out, but back from the anterior

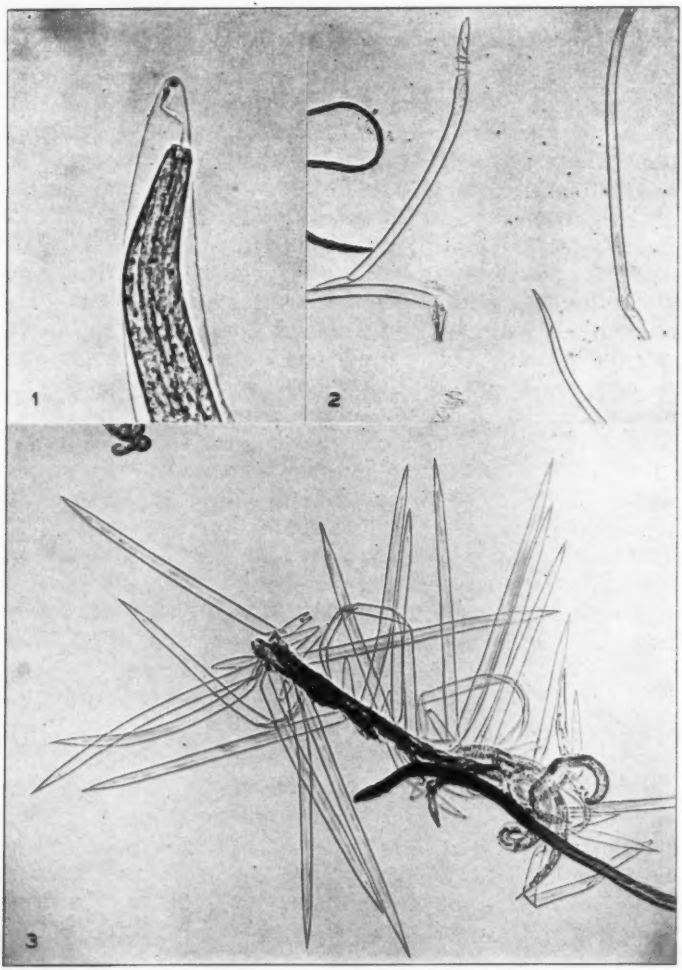


Fig. 1 Enlarged view of anterior end of *Haemonchus* larva showing provisional buccal capsule of 4th stage, within first parasitic sheath. Cuticular lining of 3rd stage esophagus is still attached to the old mouth. Occasionally on ecdysis this region of sheath is invaginated, due to efforts of the larva to free itself from the esophageal cuticula.

Fig. 2. Cast sheaths, first parasitic ecdysis of *Haemonchus*, the usual point where larva breaks through visible a slight distance from anterior end. Not infrequently this anterior portion breaks off completely as a temporary "cap" for the 4th stage larva.

Fig. 3. First parasitic sheaths of *Haemonchus* tangled on piece of sterile debris in culture tube.

(Photomicrographs by J. A. Carlile)

end about 1/8th of its length (Fig. 2). Occasionally the anterior end of the old sheath is invaginated, due to the effort of the larva to pull away from the cuticular lining of the old esophagus, shown in Fig. 1, but usually the cast sheath is a clean shadow of the 3rd stage form. If by chance a cotton fibril or other piece of sterile debris happens to be present in the culture tube, the sheaths are caught in numbers about it, as in Fig. 3.

According to Veglia, 70 per cent of the larvae may be free 4th stages by the 48th hour after infection of the sheep. In vitro, although ecdysis is occasionally observed within 48 hours, the period is longer for most of the larvae. Up to now, 70 per cent by the 4th day would classify as an exceptional in vitro result, but ecdysis has been observed to that degree in one tube examined on the 4th day.

4. If the young 4th stage larvae are not in media which contain appropriate nutriment they soon die. Generally speaking the exsheathed 3rd stage larvae introduced into culture tubes are very hardy. If parasitic development does not occur they may persist at incubator temperatures for several weeks, though in gradually decreasing numbers (see Graph 1a), growing more and more pale as the food stored in their intestinal cells is used up. This is a familiar observation concerning infective larvae of strongyloid forms, especially hookworm. The newly emerged 4th stage gives evidence of being physiologically distinct from the 3rd stage in its inability to survive without exogenous nutriment under the same conditions. As the studies here reported do not deal with nutrient media, the paradoxical criterion gradually arose that—barring losses due to contamination or specific toxic factors such as sodium chloride or strong liver extract—good conditions for ecdysis and high death rates ran more or less in parallel.

This ability of the 3rd stage larvae to make the transition to the 4th stage apparently entirely on their endogenous food reserves has proved a fortunate circumstance, inasmuch as it has permitted distinguishing factors which induce development into parasitism, from the factors favorable to further growth afterward. The degree to which ecdysis takes place thus becomes a measure of the favorableness of the environment for parasitism, and of the conditioning factors being tested.

EXPERIMENTAL RESULTS

Salt Solutions.

Two experiments are illustrated. Exper. 1, in two parts, indicates the relative desirability of Tyrode, Ringer, and physiological salt (normal saline) solutions in various dilutions, as tested in unsealed tubes; Exper. 2 deals with Tyrode solution alone in sealed tubes.

The Tyrode solution used (Parker, 1938) contains 8.0 gm. sodium chloride, 0.2 gm. potassium chloride, 0.2 gm. calcium chloride, 0.1 gm

magnesium chloride, 1.0 gm. sodium bicarbonate, 0.05 gm. sodium acid phosphate, and 1.0 gm. glucose in one liter of distilled water. In making up Tyrode, if each ingredient is dissolved in the water before adding the next, no cloudiness results. Sterilization is by candling through a Berkefeld N. The three-salt Ringer solution (Parker, 1938) can, on the other hand, be sterilized by autoclaving and contains 9.0 gm. sodium chloride, 0.42 gm. potassium chloride, and 0.25 gm. calcium chloride in one liter of distilled water. The normal saline used was autoclaved 0.85 per cent sodium chloride.

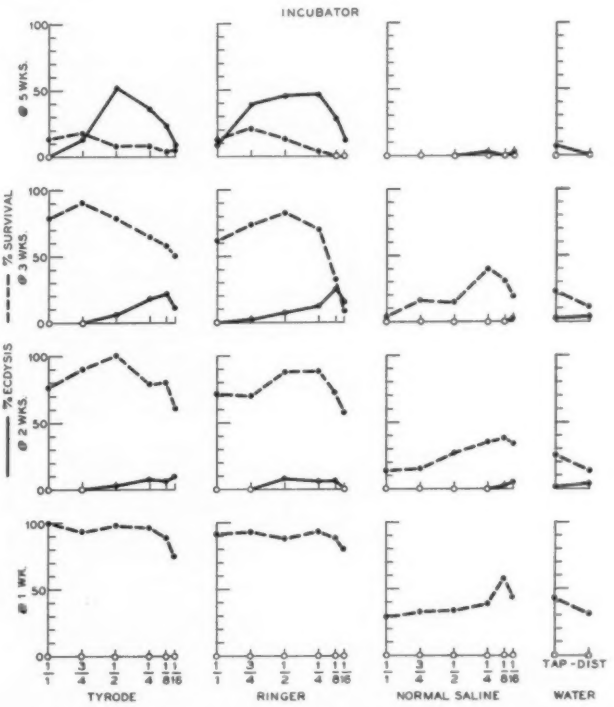
In the first part of Exper. 1 comparisons of the salt solutions and their dilutions are made on the basis of the amount of parasitic ecdysis occurring with *Haemonchus* larvae in the incubator at 38 1/2° C, in the second part, by the degree of survival of the larvae in the refrigerator at 5° C, a temperature at which ecdysis has not been observed.

Exper. 1A. To small test tubes, 100 × 10 mm, were added 2 ml. amounts of Tyrode, Ringer, and sodium chloride solutions at the usual 1/1 physiological concentration, and dilutions of 3/4, 1/2, 1/4, 1/8. and 1/16. Tubes with similar amounts of tap water and distilled water were used as controls. These dilutions produced a series in which the salt concentrations were respectively 160, 120, 80, 40, 20, and 10 millimols per liter. (Normal saline at 0.85 per cent represents 145 mM/L. Its dilutions were made to reproduce the above millimolecular series, except full concentration which represented 145 rather than 160.) The tubes were inoculated with an average of 55 sterile, ex-sheathed 3rd stage *Haemonchus* larvae, and closed with a cotton plug but not otherwise sealed. There was some evaporation from the tubes during incubation, and those continuing in the experiment after the 2nd week, and also after the 3rd week, were opened with sterile precautions and about 1/2 ml. distilled water added to restore original depths of approximately 35 mm.

At the end of 1, 2, 3, and 5 weeks the larvae in each tube were examined and counted, as were the empty sheaths. The larvae, being poor swimmers, are always in the bottoms of the tubes with the sheaths, and all can be withdrawn in a single drop. The percentages of larvae which had undergone parasitic ecdysis (i.e., number of sheaths in relation to whole number of larvae in tube) are shown in Graph 1A, as well as the percentage of surviving larvae at each examination. The results are plotted for the 80 individual tubes, not as averages.

No ecdysis was found in any tube the first week, but there was a perceptible beginning by the end of the second week, absent however in the 1/1 and 3/4 concentrations of Tyrode and Ringer, and in all but the most dilute saline. Ecdysis in saline, tap water, and distilled water, did not later exceed the minute amount apparent at two weeks.

By the end of the 3rd week, while 1/1 Tyrode and Ringer still showed no sheaths, there was an increase to over 20 per cent in the 1/8 dilutions. This had further increased but little by the end of the 5th week in the most concentrated and dilute, but in the mid-range of dilutions from a third to a half of the larvae had emerged as 4th stage forms. The relatively good ecdysis rates occurring sooner in the 1/8 dilutions of Tyrode and Ringer solutions are suggestive of results reported by Hogue (1919) who found that diluted Locke-Lewis solutions acted as a stimulant for tissue cultures. The fact that ecdysis rates at Tyrode and Ringer dilutions more nearly isotonic for *Haemonchus* larvae are eventually greater in degree than at the hypotonic 1/8 dilution is further suggestive of Miss Hogue's observation that in hypotonic Locke-Lewis solution tissues grew more rapidly but did not live so long as in isotonic media.



Graph 1A. Comparison of Tyrode, Ringer, and sodium chloride solutions in unsealed tubes in production of first parasitic ecdysis of exsheathed infective *Haemonchus* larvae. The millimolar concentrations of the Tyrode and Ringer dilutions range from 160 at 1/1, to 10 at 1/16; "normal saline" dilutions are arranged to parallel these, except 1/1 which is 145. Controls in tap and distilled water.

These young 4th stage larvae soon died, a fact reflected in the survival curves in Tyrode and Ringer solution, which represent 3rd stage larvae which have not yet undergone ecdysis. The poor survival of the larvae in saline is obviously unrelated to ecdysis, representing instead the marked toxicity of sodium chloride without the benefit of antagonistic ions, as is usual with biological material. The marked decrease in number of survivors at one week, with all dead in 5 weeks, is also true of the controls in tap water and distilled water.

Exper. 1B. As a parallel to Exper. 1A, a duplicate set of tubes was similarly inoculated from the same batch of larvae and placed in the refrigerator at 5° C. Evaporation from these tubes was slight, scarcely reaching at the end of 3 months that in the incubator after 2 weeks. Because of the known ability of *H. contortus* to remain alive under refrigerated conditions, additional tubes were included so that examinations could be made after the 5 week period.

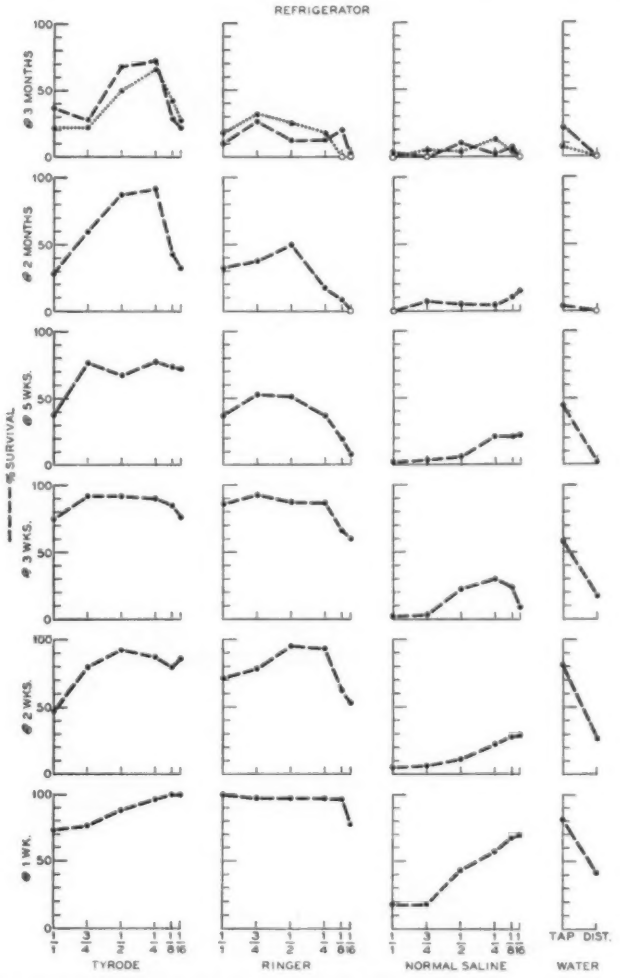
Due to the sluggishness of larvae examined while cold, the tubes were removed from the refrigerator 1 to 3 hours before examination. (See below the special treatment given a second set at the 3 month interval.) Neither ecdysis nor development preliminary to ecdysis was observed in any tube. The data on survival are plotted for the 140 individual tubes in Graph 1B.

Even by the end of the first week the death rate in normal saline was greatly increased, over either Tyrode or Ringer, although a small percentage lived for 3 months. Survival in general was better in the more dilute sodium chloride than in the "normal" concentration, although distilled water alone is an improvement for a period. Larval *Haemonchus* evidently better resist the hypotonic action of pure water in the first few weeks, than the toxic sodium ions in most of the saline dilution. Judged by the survival data the laboratory tap water* is so much better that it classifies as the substitute, which it frequently is, of a dilute "balanced" salt solution, such as Ringer.

Between Tyrode and Ringer solutions there is little distinction in the first 3 weeks' results, but there is at 5 weeks and thereafter greater survival in the Tyrode. Experience gained in other tests has indicated that this advantage of Tyrode as compared to Ringer solution, which shows more distinctly in Graph 1B than in Graph 1A, is on the whole characteristic with *H. contortus*.

*This water was from 200 foot wells. A routine commercial analysis made during the course of these studies showed, in grains per gallon: "total hardness 3.1 (temporary 1.9, permanent 1.2); total (bicarbonate) alkalinity 4.7 (both hardness and alkalinity in terms of $CaCO_3$); chlorides (Cl) 0.8; sulphate (SO_3), less than 1.5; iron, none; pH 6.7." Tap water, autoclaved, is used during the exsheathing and sterilizing of the infective larvae. Baermann isolations are done with tap water at body temperature.

While survival was anticipated at 3 months, it was a surprise to find it so good in Tyrode. To test whether larvae stored this long at low temperatures would respond to in vitro conditions to undergo ecdysis, a set of the tubes still remaining was examined in the following manner. A new series of tubes was prepared, 2 ml. each, with a Tyrode-liver extract medium know to be effective in producing ecdysis (the 0.10 gm. per ml. concentration illustrated in Exper. 3 was used). A



Graph 1B. Survival of exsheathed infective *Haemonchus* larvae in graded dilutions of sterile Tyrode, Ringer, and normal salt solutions in refrigerator, 5° C, with controls in tap and distilled water.

set of the refrigerater tubes was then opened under sterile conditions, and larvae from each were transferred to Tyrode-liver extract medium. The new tubes were then incubated for 4 days and examined. An insufficient number of live larvae remained from the original Ringer, saline, and water sets to give a test, but from the 6 original Tyrode tubes ecdysis had taken place in from 3 to 37 per cent (average 21 per cent) of the larvae still alive on the 4th day of incubation (which represented 2 to 16 per cent, average 7 per cent, of all the larvae, dead and alive, in the tubes). Reference to Exper. 3 will show that a similar Tyrode-liver extract concentration in that experiment produced 30 per cent ecdysis, with 70 per cent of the larvae surviving, on the 4th day. This demonstration of ability to undergo ecdysis after refrigeration thus attested fairly good physiological condition of the larvae, and biologically confirmed the survival curve at 3 months in Tyrode solution.

The results of the two parts of Exper. 1 are in essential agreement. Judged either by ecdysis at the end of the first parasitic (3rd larval) stage in the incubator, or simple survival of exsheathed infective larvae at a temperature (refrigerator) too low to permit ecdysis,—

1. Balanced salt solutions, such as Tyrode and Ringer, present more favorable conditions than sodium chloride solution alone, tap water, or distilled water.

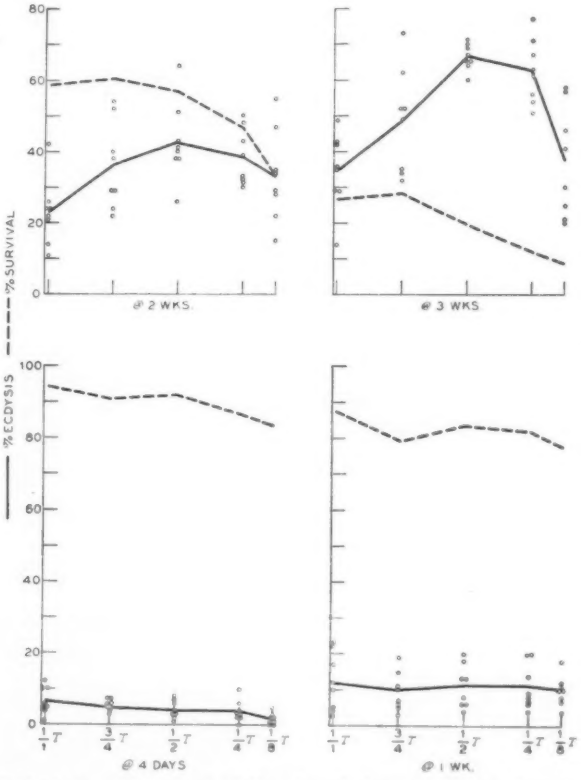
2. Balanced salt solutions at the physiological concentrations isotonic for mammalian or bird tissues offer a distinctly less favorable environment for this parasitic nematode than a range of dilutions. With sodium chloride alone in solution, its marked toxicity is decreased with decreasing concentration.

3. The production of parasitic ecdysis under the conditions outlined indicates that the inclusion of liver extract in the culture media suggested by Glaser and Stoll (1938c) is not necessary to bring about the initial phase of parasitic development culminating in ecdysis, although it may play a rôle. In the present experiment some ecdysis occurred even in distilled water. In such an exceptional environment it may have been made possible by substances set free into the water through the early death of many of the inoculated larvae (or by the trace of tap water in the inoculating drop, 0.075 ml. from the final tap water wash). Ecdysis production without the aid of liver extract is nevertheless a delayed phenomenon. In the experiment with unsealed tubes it had not occurred in one week, had scarcely begun at 2 weeks, and in the most favorable solutions, Tyrode and Ringer, was not very apparent until sometime after the 3rd week.

4. There is no indication that the pH affected the degree of ecdysis. In Exper. 1 the salt solutions were not adjusted, inasmuch as a test of

a range of pH requires adding buffer, which tends to interfere with the direct test of the salt solution ingredients alone. The Tyrode was somewhat alkaline (8.0-8.6), Ringer, saline, and water slightly acid (6.2-6.8). It is true that ecdysis occurs in media as acid as pH 3.0 (Glaser and Stoll, 1938c). But the idea has had to be modified that the beginning of parasitic development of *Haemonchus* in vitro requires a strongly acid environment, despite the fact that the abomasum of the sheep, on the mucosa of which this stage occurs in nature, is markedly acid (3.5-3.9).

Exper. 2. Various tests have been undertaken to determine whether the good ecdysis rates of the balanced salt solutions of Exper. 1A could not be produced more promptly. Exper. 2 illustrates a successful, reproducible result. In this experiment Tyrode solution alone was



Graph 2. Ecdysis in sealed tubes of Tyrode solution at dilutions from 1/1 to 1/8, at 4 days, and 1, 2, and 3 weeks. The individual results for each of 8 tubes at each examination show the variation from the mean value, full line. The broken line shows the mean survival rate in the same tubes.

used in concentrations from 1/1 to 1/8, the tubes (100 × 10 mm.; with 2 ml. solution each) were pre-incubated 24 hours before inoculation with an average of 175 *H. contortus* per tube, and sealed with "Parafilm" before incubation. Graph 2 shows the results with their averages of 160 individual tubes, expressed as the percentage of larvae in each tube which had undergone ecdysis at the 4 examination periods, at 4 days and 1, 2, and 3 weeks. The average percentage of surviving larvae at each examination is given as a dash line, the survival rate for individual tubes, which showed about the same amount of variability as the rates of ecdysis, being omitted to simplify the graph.

Under conditions of pre-incubating and then sealing the tubes, there was an acceleration of the ecdysis production as compared to Exper. 1A. It had begun by the 4th day, had doubled in a week, and showed significant increases at 2 and 3 weeks. The beneficial effects of dilutions of the usual Tyrode concentration were not discernible before the 2nd week. By the 3rd week the Tyrode series shows markedly smaller ecdysis values at 1/1 and 1/8, better at 3/4 and 1/4, and in 1/2 Tyrode concentration the 8 tubes gave individual results from 60-71 per cent, with an average of 66. Except that it took between 2 and 3 weeks to reach these ecdysis rates, the mid ranges of the Tyrode dilutions were adjudged to have produced excellent results.

Whether Tyrode is the best balanced salt solution for use in producing parasitic ecdysis is being further investigated. Preliminary observations indicate that the glucose contained in it does not play a significant rôle, for it can either be omitted or increased to quadruple concentration, without modifying the degree of parasitic ecdysis produced. Some tests, the inconsistencies of which have not yet been resolved, suggest the possibility of securing sufficiently good ecdysis with balanced salt solutions alone, perhaps comparable in shortness of time and completeness to the ecdysis values now obtainable with a supplement of liver extract such as illustrated in the next experiment.

LIVER EXTRACT

While parasitic development of infective *Haemonchus larvae* can be initiated in their absence, certain liver extracts have been found to accelerate this biological process. Glaser and Coria (1933) cultured *Paramecium caudatum* free from other living microorganisms with the aid of commercial Lilly liver extract No. 343, and also with aqueous extracts made from fresh livers. Trager (1935) successfully cultured mosquito larvae under sterile conditions with the same Lilly extract. On comparative test the effectiveness of various concentrations of Lilly liver extract No. 343 (made from swine livers), in initiating parasitic development of *H. contortus* larvae cannot be distinguished from the effectiveness of Tyrode or Ringer solution alone. With aqueous

extracts made in the laboratory from certain fresh livers, better results have been secured. Despite many experiments, with emphasis upon care in processing and standardization of tests, extracts from sheep, lamb, and calf livers showed great variability in potency. The reason for this has not yet been determined. Rabbit liver extract showed some potency, swine liver extract practically none, both derived from animals not natural hosts of *Haemonchus*.

More recently an extract prepared from Difco Bacto-Liver, when used in certain concentrations, has been uniformly successful in producing ecdysis more promptly. This extract can be titrated in terms of the amount of whole liver from which it is derived and has been found to show a similar concentration of effective substance in different lots purchased, even when the initial stock solutions are first made up at differing concentrations.

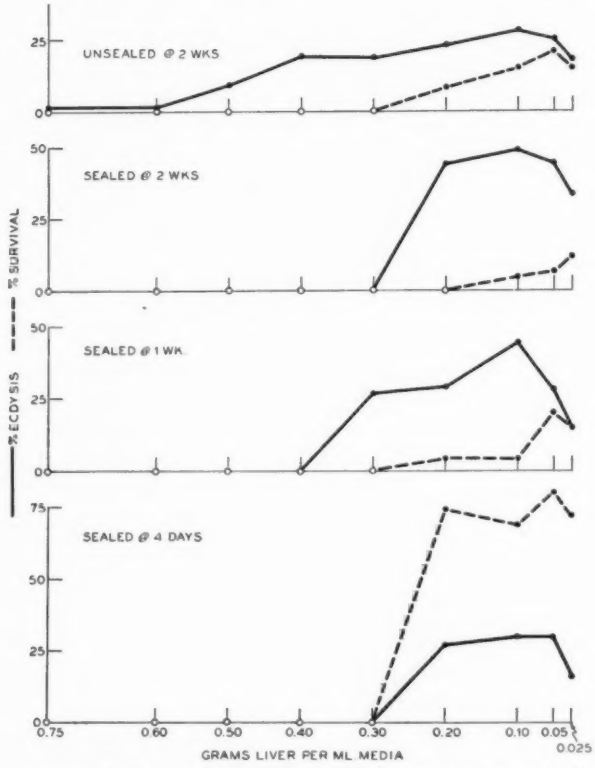
Bacto-Liver is bovine whole liver powder, 27 grams of which are stated to be the equivalent of 100 grams fresh liver. The aqueous extract has been prepared as follows: Distilled water is added gradually to 27 grams of liver powder to make first a paste and then a suspension, employing in all 173 ml. water. If acidity is not as much as pH 5.0, sufficient 3 per cent H_2SO_4 is stirred into it to reach this value. The mixture, in a covered dish, is allowed to infuse for two days in the refrigerator, with occasional stirring. It is then boiled to coagulate the proteins, and these are removed by pressing out through gauze and filtration through paper. The volume is brought to 100 ml. at this point, by further evaporation if necessary. The extract, rich brown in color, is then tubed, autoclaved, and refrigerated until used. If the boiling and protein removal have been inadequate, considerable amounts of fine precipitate will form in the tubes on cooling, the presence of which in experimental tubes causes difficulties in larval examinations. To obtain clear extract the supernate may be passed through a Berkefeld candle, or the extract refiltered through paper and again autoclaved. The making of the extract, as outlined, follows the procedure used for fresh livers, except that for the latter the amount of water employed in the first step (infusion) is equivalent in weight to the ground liver used. The sterile extracts are characteristically of pH 5.0-5.6, with 1 ml. extract representing 1 gm. original liver, and a salt concentration (calculated as NaCl) of about 80 mM/L.

A representative experiment illustrates the early increase in ecdysis produced in the presence of liver extract, and the range of extract concentration found most favorable.

Exper. 3. This was arranged to test the potency of the liver extract in graded amounts from 0.750 to 0.025 gm. original liver per one ml. medium made with a net concentration of 3/4 Tyrode solution (120

mM/L). The medium at each concentration was adjusted to pH 5.0 under sterile conditions, using N/1 and N/10 HCl. After adjustment the medium was distributed in 2 ml. amounts in 100 × 10 mm. tubes, and to each of these was then added an average of 80 sterile *Haemonchus* in a small drop of 1/8 Tyrode solution. "Unsealed" tubes had a cotton plug only; the others were covered in addition with the "Parafilm" seal. The individual results (not averages) of 36 tubes examined after 4, 7, and 14 days incubation at 38 1/2° C are shown in Graph 3.

In sealed tubes containing extract representing 0.40 grams or more of liver per ml. media, the larvae all died before ecdysis. In such cases, with a concentration of liver extract so great as to be toxic, the dead larvae are stained a deep brown, some nearly black. It is apparent that the optimal concentration of liver extract is in the range 0.20-0.05 gm. per ml. media, a usual finding with extract made as described from



Graph 3. Titration of liver extract (grams original liver per ml. media) in production of first parasitic ecdysis of *Haemonchus contortus*.

Bacto-Liver. One tube at the 7 day examination showed appreciable ecdysis at 0.30 gm., with all the larvae then dead. Despite such an aberrant tube at the margin of the favorable range, which not infrequently occurs, the results in general are highly consistent. The increased ecdysis at 7 days, over that at 4 days, was accompanied by a similar increase in the death rate, the results at 7 and 14 days not differing essentially. Survivors were exclusively larvae which failed to develop. Dead young 4th stages had shown no evidence of growth. This failure to survive is uniformly the case and is considered evidence that liver extract and Tyrode solution do not contain materials utilizable as food for parasitic growth, even though in combination they furnish an environment in which accelerated development into the 4th stage occurs.

The upper part of Graph 3 shows the results of tubes left unsealed, and examined at 2 weeks. Parasitic ecdysis here took place over a wider range of concentration of liver extract. In the four concentrations tested at 0.200 to 0.025 grams liver per ml. medium the unsealed tubes did not reach levels as high (19 per cent less on the average) as similar concentrations in sealed tubes. At 0.75 to 0.40 from a minimum to moderate amounts of ecdysis occurred in unsealed tubes, when, in contrast, it was absent from the sealed, although in both sets at these concentrations all larvae were dead at the end of the 2nd week. The most favorable concentration—0.10 gm. liver per ml. media—was alike in both sealed and unsealed tubes.

There is clearly enhancement of ecdysis here by liver extract, over the results with Tyrode solution alone in either Exper. 1 or 2. Contrasting the unsealed tubes at 2 weeks, Exper. 1A showed the mere beginning of ecdysis with most of the 3rd stage larvae still alive; whereas Exper. 3 showed good ecdysis values, although most of the larvae, whether 3rd or 4th stages, were dead, indicating virtual completion of the test. Contrasting the sealed tubes in Exper. 3, the values with optimal amounts of liver extract are greater at 4 days than Exper. 2 with Tyrode shows in any tube at any dilution in 7 days; by the end of 2 weeks without liver extract there are some values comparable to the values in Exper. 3 at 1 week. The final amount of ecdysis at 2 weeks with liver extract compares approximately with the mean value of 3/4 Tyrode solution in Exper. 2 at 3 weeks.

As a part of Exper. 3, not shown graphically, a test was made of the same liver extract after re-autoclaving. Using stock extracts at pH 5.0-5.6, different test samples of which had been re-autoclaved successively for 1, 3, 7, and 15 days, full potency was demonstrable. Extract re-autoclaved once at pH 7.0 retained its potency; extract re-autoclaved once at pH 8.0 showed some loss in effectiveness.

ECDYSIS RATES IN RELATION TO TUBE SIZE, AMOUNT OF MEDIA, AND NUMBER OF LARVAE INOCULATED

As already noted there are differences in the rate and amount of parasitic ecdysis produced in sealed and unsealed tubes. This would appear to hinge primarily on the question of oxygen tension, and to the extent that this is true such variables as the size of tube, amount and depth of media, and number of larvae inoculated would all play a rôle in determining the optimum conditions. Expers. 1-3 have already made clear that contact of the media with free air in unsealed tubes represents a less favorable ecdysis-producing environment than when the tubes are sealed. (See also Exper. 4.) Shallower depths of media, devised for either large or small tubes (i. e., differing volume-depth relationships) accentuate the poorer parasitic environment of relatively large air volumes in contact with small volumes of media. In the other direction, a few cases have occurred in which, using small tubes, sealed, nearly full of media and thus with very little volume of air above the surface, inoculations with large numbers of larvae have resulted in their death, apparently from asphyxiation. At least the tubes on examination at 4 days and later contained only dead larvae and no sign of ecdysis, although free from contamination, and with the same media in other tubes permitting ecdysis.

For most experiments tubes have been employed of 100 × 10 mm. size, in which 50-200 larvae have been inoculated into 2 ml. media. The tubes have been selected for uniformity so that the resulting depth of media is about 35 mm. If such tubes with 2 ml. media are contrasted for ecdysis with 3 ml. media, no clear difference in results with similar numbers of larvae has been demonstrable. Thus in an experiment with Tyrode-liver extract solution it was possible to contrast the degree of ecdysis production in 54 tubes with 2 ml. against 43 with 3 ml.; average ecdysis rates in the former were 23.3 per cent, in the latter 22.7 per cent, evidencing no difference in this range.

Exper. 4. When 2 ml. and 4 ml. amounts are contrasted with varying numbers of larvae, the rôle of the numbers of *Haemonchus* introduced and the interrelationships of the media volume and air volume in the sealed tubes are involved. In Exper. 4, a 0.1 gm. liver extract-3/4 Tyrode medium at pH 5.0 was used, with special care in selection of tubes so that 2 ml. in Set A reached a depth of 35 mm., 4 ml. in Set B, 70 mm. They were inoculated in parallel with graded number of larvae per tube, from less than 100 to over 2000. The results of examinations at 4 days are shown in Table I. In both the A and B sets there is in general increasing degree of ecdysis with increased numbers of larvae. However, in inoculations of the same general size there is

TABLE I

ECDYSIS PRODUCTION WITH *H. contortus*, USING GRADED NUMBERS OF LARVAE IN SEALED 100 × 10 MM. TUBES, WITH 0.1 LIVER EXTRACT-3/4 TYRODE MEDIA, PH 5.0, IN 2 AND 4 ML. AMOUNTS, PRODUCING DEPTHS OF 35 AND 70 MM. INDIVIDUAL TUBE RESULTS AT 4 DAYS

Set A. 2 ml. media, 35 mm. depth			Set B. 4 ml. media, 70 mm. depth		
Number of completed ecdyses (sheaths)	Ecdyses	Larvae alive	Number of completed ecdyses (sheaths)	Ecdyses	Larvae alive
	per cent	per cent		per cent	per cent
26	27	92	6	21	83
179	37	72	94	16	72
466	53	93	298	30	78
1451	51	84	975	44	82

increased ecdysis in the 2 ml. as contrasted with the 4 ml. amounts of media.

Exper. 5 A variety of experiments has been undertaken to explore these relationships, which are still unsatisfactorily understood. A synopsis of one such, Exper. 5, is given, in which 0.1 gm. liver extract-3/4 Tyrode medium at pH 5.0 was used. In this experiment 100 × 10 mm. tubes (total volume 5 1/2 ml.) with from 1/2 to 4 ml. medium, were compared with 100 × 13 mm. tubes (total volume 10 ml.) with from 1 to 7 ml. medium, and with 150 × 13 mm. tubes (total volume 14 ml.) with from 1 to 12 ml. medium. Half were inoculated with an average of 105 larvae, a parallel series with 390 larvae per tube, for examination at 4, 7, and 14 days. Due to some sealed tubes developing contaminations, 93 were eventually examined, classifiable in 33 different relationships of tube size, media volume, and size of inoculation. A control set of tubes (in which 24 of these relationships were paralleled) was left unsealed and examined at 14 days.

The differing amounts of medium used in various tubes gave cross-checks on comparable depths with dissimilar volumes, and vice versa, in relation to two densities of *Haemonchus* larval population. In general throughout the series inoculations with 390 larvae provided better percentages of ecdysis than with 105. There was a lag in, as well as a decreased total, ecdysis in tubes with small amounts of medium. Less ecdysis occurred in the large tubes than in either of the smaller sizes. The unsealed tubes in each set gave inferior results compared to the sealed.

In view of the planned diversity of conditions in the experiment, it was analyzed from the standpoint of the total amount of free oxygen available to the larvae, computed for the actual count of larvae in each tube. The mean ecdysis rates of the three examinations for each tube

TABLE II

ECDYSIS RATES (MEAN OF VALUES AT 4, 7, AND 14 DAYS) IN RELATION TO AVAILABLE OXYGEN PER 100 *H. contortus*, COMPARING TUBES WITH DIFFERING RATIOS OF LARVAE AND AMOUNT OF MEDIA IN TUBES OF DIFFERENT SIZES. SEE TEXT

Total ml. oxygen available per 100 *H. contortus*	Per cent ecdysis							
	1-4	5-9	10-14	15-19	20-24	25-29	30-34	35-39
.05 - .24			1	2	1		1	1
.25 - .49	1	5	1	1	2			
.50 - .74	1	1	1	1				
.75 - .99	1	1	3					
1.00 and over	1	6		1				
Unsealed	4	9	1					

size with a given amount of medium are shown in Table II in relation to the amount of oxygen sealed in the tube per 100 *Haemonchus*. The oxygen computations were made assuming the usual amount in air, at standard pressure, with solubility of the oxygen in the medium comparable to that in water at the same temperature; "ml. total oxygen" was derived from the sum of the dissolved oxygen plus that in the enclosed air of the sealed tube.

Such an analysis is admittedly an approximation, but does to a certain extent define, for the first parasitic ecdysis of *Haemonchus*, the combined rôle of media volume, total oxygen volume dissolved in medium and present in the air in the sealed tube, and number of larvae introduced. It is immediately apparent that the best average ecdysis rates occur exclusively in the low values of oxygen available per 100 larvae. Reciprocally, the poorest ecdysis rates are missing from that same oxygen level, but are especially numerous in the highest oxygen level, classified here as 1.00 ml. oxygen or more per 100 larvae. In complete support of the latter grouping is that of the unsealed controls, the media of which were, of course, uninterruptedly in contact with free air, and which show a similar clumping of low average ecdysis rates.

This expression of the favorable ecdysis-producing environment is not peculiar to Exper. 5. As illustration it may be pointed out that the five highest ecdysis rates in Exper. 4, Table I, are in tubes whose computed oxygen values fall in the line of the smallest amount oxygen per 100 larvae of Table II.

These general relationships suggest that for best results in the production of ecdysis with *Haemonchus* a certain low oxygen tension is desirable, and that this is reached rather more readily in small test

tubes, with moderate amounts of media, sealed after inoculation. As a practical procedure in comparing ingredients and culture conditions, larval inoculations of about 100 *Haemonchus* per 100 × 10 mm. tube containing 2-3 ml. medium have been favored because they are more serviceable for intra-experimental comparisons with a saving of time in making examinations, than tubes containing large numbers of organisms. Such favored tube sizes, etc., have oxygen values which strike the middle of those specified in Table II.

DISCUSSION

The experiments here reported show that the first parasitic ecdysis of *H. contortus* may be accomplished in vitro under sterile conditions with certain definite expectations. It succeeds with balanced salt solutions, alone, such as Tyrode or Ringer, and essentially fails with unbalanced sodium chloride solution. It succeeds better with balanced salt solutions at less than the usual concentrations which are physiological for mammalian or bird tissue. The optimal range of dilution is from about 1/4 to 3/4 representing a concentration of 40 to 120 millimols per liter. It succeeds better, under the conditions of the experiments here reported, in small tubes with moderate amounts of media, and when the tubes are sealed rather than unsealed; and better with larger than smaller numbers of larvae.

This first parasitic ecdysis, while it is accomplished in balanced salt solutions alone, is accelerated with certain concentrations of an aqueous liver extract, of which the most reliable to date has been prepared from Difco Bacto-Liver.

This production of parasitic ecdysis in *Haemonchus* is a biological phenomenon, and not an extension of the chemical exsheathing process by which the larvae have been earlier sterilized. The exsheathing of the infective larva is of a larval stage with a sheath already formed and ready to cast. Parasitic ecdysis is the culmination of a "pupation," involving the formation of the new sheath at the end of a process in which morphological changes occur in the mouth parts of the larva, interpreted functionally as making it able to attack the mucosa of the ruminant 4th stomach, its normal residence. The development ending in parasitic ecdysis does not occur in larvae kept under refrigerated conditions in the same solutions in which they will undergo the process when incubated at 38 1/2° C. So far it has not been observed at room temperature, although tests have not been exhaustive on this point. Contrary to expectation, it has not been dependent upon either acid or alkaline environments, taking place in both. It occurs better under conditions of restricted oxygen tension.

From these facts it would appear that parasitism for *H. contortus* begins when there has been substituted for the free-living environment

outside the host, one in which there is a physical environment including lessened oxygen tension and a continuously higher temperature, comparable to that of the sheep abomasum, together with a certain chemical balance in the menstruum in which it finds itself comparable to that reproduced by a dilute balanced salt solution. The further fact that parasitic ecdysis as a process is accelerated in the presence of aqueous liver extract suggests that under ordinary parasitic conditions certain biological factors stimulate the reaction. The substance or substances in liver extract may play this rôle in nature, or may constitute an in vitro substitute.

It is significant that *H. contortus* completes this first parasitic ecdysis without dependence on food other than reserves stored in its own cells and then, as a young 4th stage larva, dies, if appropriate food is not available. Possibly this is a characteristic of parasites which under natural conditions reach a new biological stage of development promptly after reaching a new host. The evidence is not clear in Ferguson's (1940) culture of a metacercaria which evolves promptly to an egg-producing trematode whether the media constituents, which, besides dilute Tyrode, include serum and yeast extract, are of actual nutritive assistance to the parasite; *P minimum* like *H. contortus* completes a first parasitic "stage" in the host within less than 48 hours.

Once the fact is demonstrated that salt concentrations which are appreciably less than those isotonic for tissues prove best as a parasitic environment for *H. contortus* larvae, it is seen to be clearly related to *Haemonchus* biology . This parasite, in nature living superficially on the wall of the ruminant stomach at this stage, is to be thought of as inhabiting the lumen rather than the tissues, even though it lies very closely in apposition to the epithelial cells. Gastro-intestinal helminths in general may require such a diluted salt solution in vitro, and Ferguson (1940) has recently indicated that for the strigeid, *P. minimum*, a 5/8 Tyrode solution was the most favorable concentration. Cobb (1924) "raised the question as to the best solution strengths to keep nemas alive, noting that he had found normal saline unsatisfactory for free living nemas," but whether this was due to toxicity of sodium chloride or hypertonicity of the solution is not clear. Fenwick (1939) testing the longevity of *Ascaris suum* larvae for saline requirements found they lived longest, 5 1/2 days, in a suitably antagonized saline whose constituents represent full strength Tyrode solution, minus the buffers and glucose (Parker, 1938), with a computed millimolar concentration of 142, in contrast to the 40-120 range found more suitable for *Haemonchus*. This difference may signify that the newly emerged *A. suum* larva requires a medium isotonic with tissue fluids, which would be in harmony with the well known tissue wandering habits of *Ascaris* at this stage in its life cycle.

Of interest in this same connection is the circumstance that conditions favoring the first parasitic ecdysis of *H. contortus* in vitro are not necessarily effective for other forms. Tests with *Trichostrongylus axei* show that it does not respond as does *Haemonchus*, although the infective larvae are readily exsheathed and sterilized for test.

SUMMARY

The first parasitic ecdysis (end of 3rd larval stage) of *Haemonchus contortus* may be secured in vitro with balanced salt solutions such as Ringer and Tyrode. (Sodium chloride alone is toxic.) Better results are obtained with less than the usual physiological concentrations for tissues, the favorable range being 1/4 to 3/4 (40-120 mM/L). In incubated unsealed tubes ecdysis begins after a week, reaches its best values after a month. In sealed tubes the time is approximately halved. With the supplement of a small amount of liver extract (aqueous), ecdysis proceeds more promptly, and under appropriate conditions may occur with from a third to a half of the worms in the culture tubes in 4 days. The liver extract is titratable in terms of the amount of original liver represented per ml. of culture media. Better ecdysis rates also occur under conditions of restricted oxygen, as secured in small tubes with moderate amounts of media, pre-incubated before inoculation, and then sealed.

Distinction between factors favoring the first parasitic ecdysis in vitro and those which allow development beyond it, is possible because of the dependence of the larvae on endogenous food reserves during this initial step in parasitic life.

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BOOK REVIEWS

Essentials of Nutrition. HENRY C. SHERMAN and CAROLINE SHERMAN LANFORD. X + 418 pp., $3.50. The Macmillan Co., 60 Fifth Ave., New York, 1940.

Vitamins, What They Are and How They Can Benefit You. HENRY BORSOOK. XIII + 193 pp., $2.00. The Viking Press, New York, 1940.

The Fundamentals of Nutrition. ESTELLE E. HAWLEY and ESTHER E. MAURER-MAST, and discussion of the dietary management in specific conditions by Samuel H. Basset, Walter R. Bloor, Samuel W. Clausen, Ray G. Daggs, Cyril M. Day, Ward L. Ekas, Fred S. Gachet, Paul H. Garvey, Charles B. F. Gillels, Jerome Glaser, E. Henry Keutmann, Lawrence A. Kohn, Irvine McQuarril, Harold B. Pierce, Frieda S. Robscheit-Robbins, Winfield W. Scott, W. J. Merle Scott, Henry L. Segal, Doran J. Stephen, with a foreword by John R. Murlin. XI + 477 pp., $5.00. Charles C. Thomas, Springfield, Illinois, 1940.

These books, which reached the reviewer's desk during the same week, are strikingly similar in their accent on the practical, health, and economic aspects of nutrition. They are, however, addressed to different audiences.

While BORSOOK, the Professor of Biochemistry at the California Institute of Technology, is also a physician, his major interests were theoretical: endogenous nitrogen metabolism, calorigenic effects of food, oxidation-reduction potentials. The appearance from his pen of a sprightly written, highly practical book, with direction on how to feed one's self at home and restaurant is an unexpectedly pleasant surprise. His emphasis on the use of middlings (now fed to cattle, not dependent on dietary B complex) as human food as source of vitamin B is timely.

SHERMAN'S book differs from his previous well-known text books on nutrition in giving less space to the biochemistry of food and nutrition and more to the recognition that "one must observe an economic limitation" in the choice of food, and on "how to make nutritional knowledge more effective."

A special feature of SHERMAN'S book is the renewed emphasis on the importance of milk in the dietary, which the readers of this review may wish to remember: "Milk is the one food . . . for which there is no fully satisfactory substitute". "Milk contains more calcium than does . . . saturated lime water". "Milk has among its nutritional characteristics a uniquely well-balanced mineral content. The only important exception (so far as known) are iron and copper which nature conveys

from mother to young largely before birth". "The modern farmer feeds his milch-cows vitamin-rich rations the year around . . . and commercial winter milk is more like summer milk, than the scientific literature might lead one to expect." Most people appear to be alike in needing milk, but they differ in their ability to get the milk they need; a major problem is how to place milk within the reach of all.

Both, SHERMAN'S and BORSOOK'S, books are highly recommended to those wishing to improve their nutritional status—and who does not? SHERMAN'S book is evidently intended for the home-economics class room, containing exercises, selected text-book references, and index; BORSOOK'S book is evidently intended for the literate rapid reader who wishes to stream-line his diet by an hour's reading in an easy chair, and it has no references, or index.

The book by HAWLEY and MAURER-MAST is in a class by itself—intended for the medical student in the habit of taking it straight. This is a highly competent and authoritative logically useful classification of an enormous body of nutritional knowledge needed by the medical student and practitioner. The economic aspects—relative cost of the various vitamins—are not overlooked. A great deal of work has gone into the construction of the many tables. The authors deserve warm congratulations on their original contribution to the nutritional text-book literature.

SAMUEL BRODY.

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The Proceedings of the Second Symposium of the Society for the Study of Development and Growth, held at Salisbury Cove, Maine from June 20 to 25, 1940, will be ready for distribution by May 1st. They will appear as Supplement to Volume IV of GROWTH and be sold as a separate volume of about 175 pages and 28 illustrations. Pre-publication price for members of the Society for the Study of Development and Growth $1.50; all others $2.50. Price after May 1st, for members $2.00, all others $3.00.

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THE THIRD GROWTH SYMPOSIUM

The annual symposium of the Society for the Study of Development and Growth will be held between July 7th and 11th at Dartmouth College. Detailed announcements will be sent out later.

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